STUDIES

IN

MARINE NATURAL PRODUCTS

A Thesis submitted in partial fulfillment
of the requirements
for the degree of
Doctor of Philosophy in Chemistry
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by

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CONTENTS

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ABSTRACT

Two new β-carboline alkaloids were isolated from the bryozoan *Cribricellina cribraria*. One of these, 1-vinyl-8-hydroxy-β-carboline, was the major cytotoxic component of the extract, while the other had a novel sulphone structure. Several other known β-carboline alkaloids were also isolated from the extract. To investigate structure-activity relationships in these compounds, several derivatives of the major alkaloid were prepared and a number of 1-substituted β-carboline alkaloids synthesised. Nmr spectroscopic studies of these compounds were carried out and previously published ^{13}C nmr data for some of these compounds revised. Homarine was isolated as the major water soluble component of the extract and the sterol composition examined. Known β-carboline alkaloids were isolated from the related bryozoan *Margaretta barbata*.

The known biologically active compounds, girolline and hymenialdisine were isolated from the sponge *Axinella* sp. 2. As some discrepancies between the experimental and published data on hymenialdisine were noted, an X-ray crystal structure analysis was performed. Oroidin, homarine and taurine were also isolated from the extract and the sterol composition found to consist exclusively of ring-contracted A norstanols.

An extract of the sponge *Stylopus australis* was found to contain a new sterol sulphate and two derivatives of this compound were prepared. The full assignment of the ^{1}H nmr spectrum of the sulphate was achieved with the aid of COSY, nOe, HETCOR and XCORFE nmr experiments. The glyceryl ether, chimyl alcohol was also isolated from the sponge.

An extract of the related sponge, *Hymedesmia* sp. 1 was examined and rhodoic acid isolated as the major water soluble component, along with homarine. The sterol compositions of *Stylopus australis* and *Hymedesmia* species 1 and 2 were examined for comparative purposes.

Studies on an extract of the sponge *Hymeniacidon hauraki* led to the isolation of a new furan fatty acid. The sterol corbisterol and its peroxide were isolated from one sample of the sponge and this led to an examination of infraspecific sterol variation in this species.

A screening procedure of biologically active natural product extracts has been developed. The procedure involves examining the chromatographic behaviour of the biological activity of an extract and is designed to detect known biologically active compounds and to determine the best means of handling extracts containing unknown biologically active components.

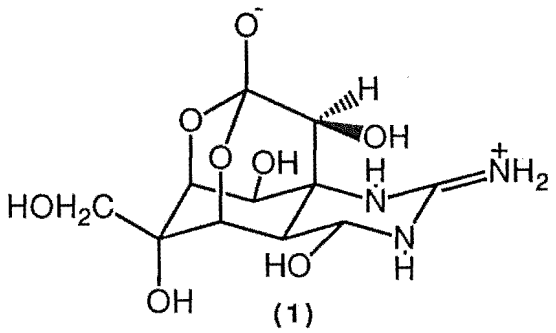
CHAPTER ONE

1.1 INTRODUCTION

Secondary metabolites, which are also known as natural products, may be defined as compounds with no clearly recognisable function in the internal economy of the organism that produces them[1,2]. The often complex structure of secondary metabolites, coupled with the great variation in secondary metabolism, both within one type of organism or between organisms, make them ideal candidates for pharmaceutical investigations[2]. Indeed, the study of natural product extracts has yielded a great number of compounds of use to man, many of which are pharmaceuticals, for example, penicillin, morphine and aspirin. To date, the majority of these compounds have been derived from terrestrial sources.

The oceans however, cover nearly 71% of the earth's surface[3] and are a vast, largely untapped chemical resource. The oceans represent a more stable environment than the land and approximately 90% of all species of living organisms dwell there[4]. All life is derived from the sea and it follows that those species that never left the oceans have had more time to adapt to their environment than their terrestrial counterparts[5]. Twenty six of the twenty eight main phyla in the animal kingdom are aquatic to some degree and eight of these phyla such as the Porifera, Bryozoa and Echinodermata, are exclusively aquatic and largely marine dwelling[5]. Statistically therefore, it is not surprising that man's attention has finally been drawn to the sea as a potential source of biologically active compounds and as a source of compounds with novel structures.

Until relatively recently, there were procurement problems involved in the study of marine organisms, both in terms of accessibility and in getting sufficient material to work with. The latter problem may still be a significant one in some cases, but with the advance of technology and in particular the advent of SCUBA diving, the ocean environment has become much more accessible to man. Some drugs from the sea are already in use, an example being tetrodotoxin (1), which is derived from pufferfish and selectively blocks the sodium channels of nerve membranes. It is used to relax muscle spasms[3] and as a probe for the study of neurophysiological mechanisms[6].



(1)

The isolation of a chemically pure substance from a crude marine natural product extract is not a simple matter. Not only is the desired substance usually part of the complex matrix of components which constitute the extract, making purification inherently difficult, but often it is also present at a low level in the organism, which compounds the isolation problem and may make it difficult to obtain enough of the chemical for characterisation or further experimentation. A compound might also be unstable when purified, or subject to chemical modification in the isolation procedure.

This introduces another problem, namely that of identifying the true source of a particular compound. A compound may be an artefact of the isolation procedure used, it may be a metabolite of the organism under study, concentrated from a dietary source or derived from a symbiotic organism. Sometimes this problem can be resolved, by, for example, changing the isolation conditions, cell fractionation or ecological studies. It has been shown for example, that tetrodotoxin (**1**) is actually produced by a symbiotic bacterium of the genus *Pseudomonas* which has been isolated from the skin of the pufferfish, and not by the pufferfish itself[7]. Tetrodotoxin has also been isolated from other marine bacteria[7]. Too often however, the actual source of a marine metabolite remains unclear. This is particularly true for sessile marine invertebrates which are subject to overgrowth by surrounding and encrusting species, or are vulnerable to invasion by microorganisms.

Over the years, routine screening of marine species for biological activity has shown that it is these sessile marine organisms that are most likely to contain compounds with promising biological activity[8]. Soft-bodied marine invertebrates such as tunicates and sponges without spicules, would seem especially vulnerable to predation[9], but many of the biologically active marine natural products isolated thus far have been from such organisms. It is thought that the animals may produce these compounds as a chemical defence against predation, overgrowth or microscopic infection[9]. In fact, a survey of the incidence of antiviral activity and cytotoxicity in sponges in the Canterbury Marine Chemistry group's collection, has shown a higher

incidence of biological activity in encroached and encrusting sponges than in the solitary species which are not competing for space[10].

Screening programmes of marine species initiated to date have varied widely in both scope and focus. Some screening is purely chemical in nature, the aim being to isolate and purify the major components from a given organism. These compounds may subsequently be assayed for biological activity[11]. This form of screening may lead to the discovery of novel chemical structures or give taxonomic or ecological information. It may also lead to the discovery of compounds with useful biological activity but it does not maximise the probability of this. Of the thousands of new compounds isolated from marine organisms with published structures, less than 5% have been investigated by pharmacologists[12]. As published structural types cannot be patented, it is unlikely that any of these compounds will be used for drug prototype development. Thus, it is somewhat ironic that the emphasis on chemistry of marine compounds has hindered the development of these compounds as potential pharmaceutical leads[12].

A more useful approach than chemistry-driven screening, is bioactivity-based screening, which employs biological assays in each step of the procedure used in the chemical isolation, in an attempt to determine the component or components responsible for the biological activity of the extract under consideration. The probability of finding useful biological activity might be further increased by targeting the collection of selected phyla which past screening has shown to contain a high incidence of species with biological activity, however this method may preclude the location of highly active species in other phyla[8].

A wide variety of biological assay methods are currently available for use in this work, including both *in vitro* and *in vivo* techniques but a given research group will only use those assay methods designed to detect the types of biological activity in which its interest lies. Advantages of *in vitro* techniques are that they are relatively inexpensive, more sensitive and faster than *in vivo* methods. However, they do not give an accurate indication of the activity that a substance will exhibit when administered to an entire organism, when such factors as the difficulty of ensuring that the compound reaches its target site become much more significant.

A large scale bioactivity directed screening programme has been initiated by the National Cancer Institute of the National Institute of Health in the United States of America, (NCI). This initiative comprises a global natural product selection

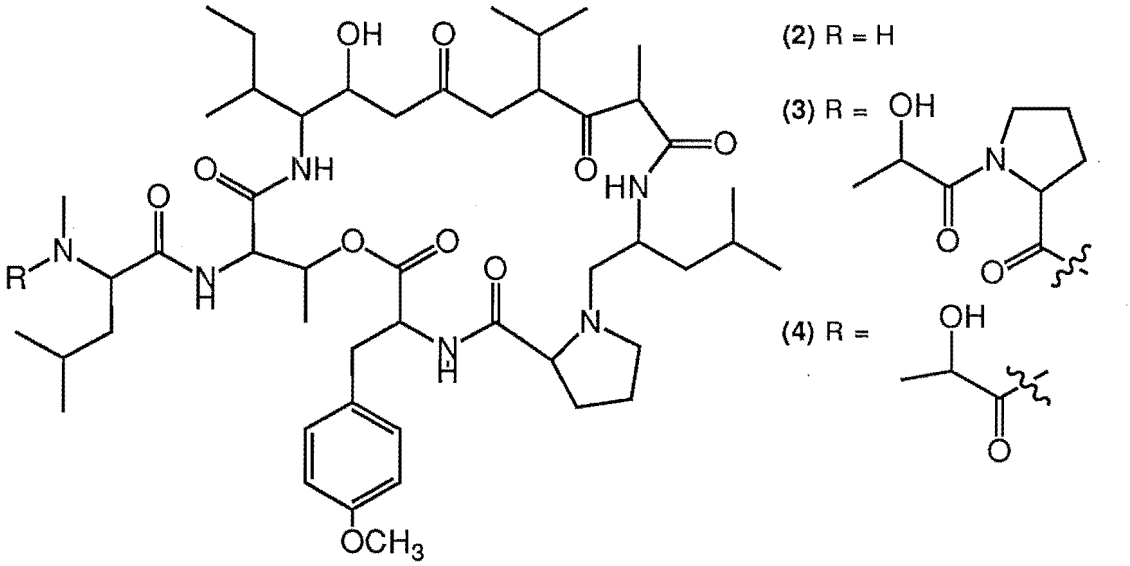
programme, including a major investigation of marine species as a source of potential new anticancer drugs. The screening programme utilises a wide variety of *in vitro* assays designed to identify compounds which show specific inhibition of particular tumour types; especially solid tumours[13].

In the past, commonly employed screening tests were based on mouse leukaemia models such as P388. This cell line is composed of rapidly dividing cells and has identified compounds of potential use against such tumours, but perhaps not surprisingly, such models are of little use in identifying agents which inhibit slow growing solid tumours such as lung and colon cancers[13].

The assays currently used by the NCI include newly developed techniques which involve the excision of actual human tumour cells and subsequent *in vitro* cloning[14]. As a single tumour is heterogeneous with respect to cell type and further histological variation occurs between different tumours of the same type, this "disease-oriented" approach requires the use of a number of cell lines, representative of each type of tumour under investigation[14].

The next testing stage, once the structure of an active compound is known is usually *in vivo* screening, which serves to define the potential of a compound for use as a drug. Factors such as undesirable side effects of a drug or deactivation by metabolic processes can be assessed.

Drugs which show promise in *in vivo* screens go on to Phase I clinical trials where the maximum dose of the drug which is non-toxic to humans is determined. The optimum dose regime against a variety of human cancers is determined in Phase II trials. The first marine natural product to be tested in human clinical trials is didemnin B (**3**), one of a series of cyclic depsipeptides (**2-4**) isolated from the Caribbean tunicate *Trididemnum* sp.[13]. These compounds show significant antiviral activity both *in vitro* and *in vivo*, in addition to the impressive antitumour activity of (**3**)[5]. It should be pointed out that in spite of the thousands of extracts screened in these programmes, only a handful of compounds derived from these ever reach clinical trial. This has not deterred research in any way; in fact research into natural products of this type is flourishing, as scientists realise their potential as pharmaceutical leads.



The isolation and identification of a biologically active component is of course only the first step in obtaining a clinically useful agent. Structure-activity relationships are an integral part of the work, as not only is it important to identify the parts of the structure that are responsible for the activity of a compound, but often it is not the isolated compound itself which is the most effective drug, but an analogue of it. Synthetic modifications of a compound may serve to increase potency, reduce toxicity or side effects or improve solubility for example. If a compound is to be of use as a clinical agent, it is important to establish synthetic routes for its production, as it is unlikely that a sufficient supply can be obtained for this purpose from the natural source.

The Marine Chemistry group at the University of Canterbury is primarily concerned with the isolation and structural determination of potential antiviral and antitumour compounds. With this goal in mind, collections of organisms have been made from northern New Zealand to Antarctica. Work has mainly focused on the study of marine invertebrates such as sponges. Although a sponge is the most primitive of all multicellular animals[3], the phylum Porifera, like those of other marine invertebrates displays a high incidence of biological activity in screening procedures, probably for the reasons of chemical defence outlined earlier. Surveys have shown that the phyla which most frequently possess antiviral activity are also those most likely to contain cytotoxic compounds[8], so a simultaneous search for these types of biological activities is justified.

The search for antiviral agents is a difficult one. Viruses are the smallest biological structures known that carry all the genetic information required for

replication and unlike procaryotes and eucaryotes, contain only one type of nucleic acid[8]. Viruses require host cells for reproduction, subverting the cell mechanisms for protein synthesis and nucleic acid replication to produce essential viral proteins and nucleic acids. Once formed, the new viral particles leave the host cell to invade others. As viruses normally invade specific cell types they are usually species specific[8]. An antiviral agent must inhibit some stage in the viral life cycle without damage to the host. The difficulty lies in the similarity between viral infected cells and normal host cells and is the reason that few antiviral agents have been developed. The University of Canterbury Marine Chemistry group employs an *in vitro* antiviral assay in its screening of marine extracts, which also gives an indication of the cytotoxicity of a compound (see below).

The terms antitumour or antineoplastic refer specifically to *in vivo* activity, while cytotoxicity refers to activity against cells cultured *in vitro*. Anticancer is a term exclusively used for results from clinical trials in humans[8]. The rationale behind the screening methods used by the NCI to evaluate potential antitumour agents has been outlined above. The range of facilities available at the NCI however is beyond the resources of many groups of researchers. Other simpler methods are often used to assess cytotoxicity. One example is the fertilised sea urchin egg assay. In this system the effects of a test compound on the mitotic division of a freshly fertilised egg are monitored. The test compound is added within five minutes of fertilisation. In a control, the first cleavage of the egg occurs after two hours. A substance is considered active if it causes 80-100% inhibition of cleavage at concentrations of 16 μg/ml or less[8]. Other simple tests used as biological activity screens are the brine shrimp and crown-gall potato disc assays. The brine shrimp assay gives a good indication of cytotoxicity, but is not specific for antitumour activity[8]. The crown-gall potato disc assay assesses the ability of a compound to inhibit crown-gall, a neoplastic disease induced in potato discs by inoculation with a bacterium, *Agrobacterium tumefaciens*[15,16]. Results show good correlation with *in vivo* P388 activity, but the reliability of the test is a problem. However, a combination of the brine shrimp and crown-gall potato disc assays has been used successfully to minimise the need for extensive *in vivo* testing in the isolation of piceatannol, the antileukaemic principle from the seeds of *Euphorbia lagascae*[17]. At the University of Canterbury, an *in vitro* P388 assay system is used to assess potential antitumour agents (See below).

1.2 BIOLOGICAL ASSAYS

Three *in vitro* biological assay systems are employed in the majority of the work described in this thesis. An outline of the assay systems used follows.

THE ANTIVIRAL/CYTOTOXICITY ASSAY

This assay determines any antiviral and/or cytotoxic properties of compounds present in a test solution and employs a BSC continuous cell line derived from African Green monkey kidney cells. Assay wells (13 mm diameter), containing a cellular monolayer are infected with either a DNA virus, *Herpes simplex* Type 1 (HSV1) or the *Polio* virus strain (PV1) which is an RNA virus. The cells are then overlaid with a methyl cellulose layer. Paper discs of diameter 6 mm are impregnated with the desired amount of test solution and air dried for twenty minutes at room temperature. The discs are pushed through the methyl cellulose overlay to sit directly on the virus infected cells. Virus and cell controls are included with each run. The cells are incubated for 24 hours at 35°C in a 5% CO_2 enriched atmosphere. Any biologically active compounds present can diffuse out of the paper disc and inhibit viral and/or cellular growth.

After incubation, an inverted microscope is used to examine the wells for the size of the antiviral and/or cytotoxic zones. Antiviral assay results are tabulated as the excess radius from the disc at which virus inhibition is observed, as in Table 1.1. Cytotoxicity is estimated from the survival rate of the BSC cells and the designation system used is the same as that used to estimate virus inhibition. The appearance of the dead monolayer cells is also noted however, and listed as a "cytotoxicity type" which is scored subjectively as in Table 1.2. This "cytotoxicity type" is generally consistent for a particular test compound or extract.

Unless otherwise stated, antiviral/cytotoxicity assay results are reported in this thesis in the form : HSV1 score, PV1 score, cytotoxicity plus the optional cytotoxicity type and the weight of sample impregnated onto the disc in micrograms. For example 2+ 2+ ww C7 20, indicates that for a sample of 20 μg impregnated onto a disc, for both HSV1 and PV1, the antiviral/cytotoxic zone is 2-4 mm excess radius from the disc edge and the cytotoxicity is over the whole well and is of type C7.

Table 1.1: Interpretation of Antiviral/Cytotoxicity Results.

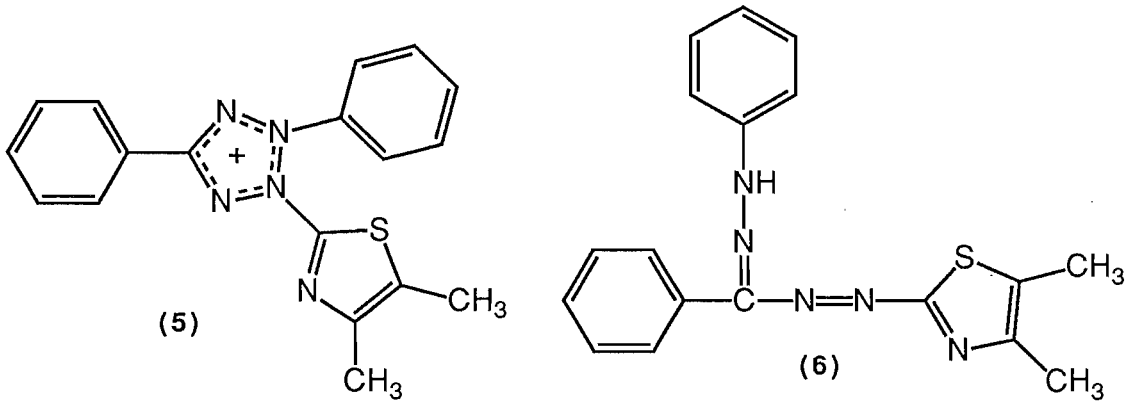
?	indeterminate activity.
-	no discernible antiviral or cytotoxic effect.
±	minor effects located under the disc.
1+	antiviral/cytotoxic zone 1-2 mm excess radius from disc edge.
2+	antiviral/cytotoxic zone 2-4 mm excess radius from disc edge.
3+	antiviral/cytotoxic zone 4-6 mm excess radius from disc edge.
ww	antiviral/cytotoxic zone over the whole well.

Table 1.2: "Cytotoxicity type" Designations.

C1	Defined nuclei.
C2	Cells are enlarged and "stringy".
C3	Cells are a mixture of rounded and elongated types. "Scruffy".
C4	Cells are rounded but separate from each other.
C5	Cells are rounded and clumped together.
C6	Cells are greatly increased in volume.
C7	Cells appear granular.
C7*	Distinct cells but appear hazy.
C8	Cells appear elongated with prominent nuclei.
C9	Cells are rounded with ragged edges and prominent nuclei.
C10	Cells are rounded and shrunken.
C11	Cells are enlarged with squared ends and form a mosaic pattern.

THE P388 ASSAY

The purpose of this assay is to determine the concentration of sample required to reduce P388 leukaemia cell growth by 50%, the IC_{50} value. The test solution is added to wells containing the leukaemia cells in a series of eight two-fold dilutions. Media, solvent, cell and positive controls are included in each assay run and the cells are incubated at 35°C for three days.



To determine the number of viable leukaemia cells present in the assay wells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (**5**) is added to the wells and these are incubated for a further four hours. Healthy leukaemia cells reduce the yellow MTT to the purple formazan product (**6**) and the amount of formazan present in each well is determined spectrophotometrically, by measuring the absorbance at 540 nm.

The absorbance is expressed as a percentage of the absorbance of the cell control which contains media, MTT and leukaemia cells but no test solution. A plot of the absorbances versus the logarithm of the sample concentrations is constructed, enabling the IC_{50} value to be determined from the antilog of the 50% value. A typical IC_{50} plot is shown in Figure 1.1.

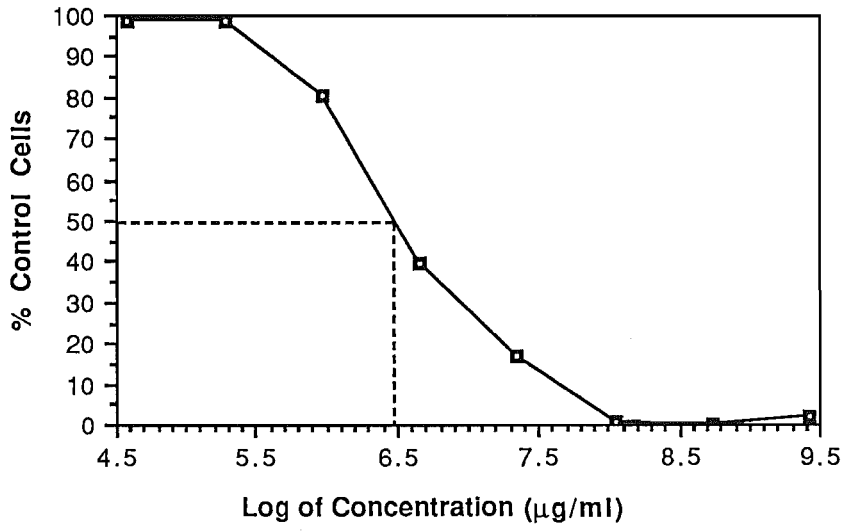


Figure 1.1: A typical plot obtained from the P388 assay results, which is used to calculate an IC_{50} value.

THE ANTIMICROBIAL ASSAY

This assay determines if a test solution inhibits the growth of bacteria and/or fungi. The organisms used are two gram negative bacteria, (*Pseudomonas aeruginosa* and *Escherichia coli*), a gram positive bacterium (*Bacillus subtilis*) and three fungi; *Candida albicans*, *Trichophyton mentagrophytes* and *Cladisporum resinae*. The organisms *Trichophyton mentagrophytes* and *Cladisporum resinae* only became available during the latter part of the work in this thesis.

The bacteria or fungi are mixed with agar and poured into a petri dish so that a "lawn" of bacteria/fungi will grow over the dish on incubation. The desired amount of test solution is impregnated onto paper discs of 6 mm diameter, which are then air dried at room temperature for twenty minutes. Antibiotic and solvent control discs are placed on the surface of the seeded agar dishes along with the test discs and the dishes are incubated at 35°C. Incubation proceeds for 24 hours for all organisms except *T. mentagrophytes* and *Cladisporum resinae* where the required incubation period is 48 hours.

After incubation, the dishes are inspected for growth inhibition caused by any antimicrobial compounds in the test solution. The size of any inhibition zone is measured as the excess radius from the edge of the paper disc in millimetres. Other visual parameters are recorded as in Table 1.3.

Table 1.3: Interpretation of the Antimicrobial Assay.

-	No inhibition of bacterial growth.
GE	Growth enhancement of bacteria.
GR	Growth retardation of bacteria.
x	Size of inhibition zone in millimetres from disc edge.
	HM Hazy margin at inhibition zone edge.
	SM Sharp margin at inhibition zone edge.
*	Slight activity, <1 mm inhibition zone.

1.3 WORK IN THIS THESIS

Work in this thesis has encompassed the major areas of interest of most natural products research; namely the isolation and characterisation of new compounds, both on a bioactivity-directed and chemistry-directed basis and the study of the components of an organism for taxonomic or ecological reasons.

In keeping with the aims of the Canterbury Marine Chemistry group, the major focus of the work has been on the biological activity of marine organisms.

Bioactivity-directed separations led to the isolation of a new β-carboline alkaloid fron the bryozoan *Cribricellina cribraria*, as the major cytotoxic component of the extract. Several minor β-carboline alkaloids were also isolated, of which one had a novel sulphone structure and some derivatives of the major alkaloid were prepared. Studies on the biological activity of the related bryozoan, *Margaretta barbata* were reinitiated and some known β-carboline alkaloids isolated.

To explore structure-activity relationships, a number of 1-substituted β-carboline alkaloids were synthesised. Nmr spectroscopic studies of these compounds and of the natural product alkaloids were performed. Attempts were made to account for the observed biological activities and to correlate these with physical parameters, so to this end, partition coefficients, Rf values and hplc retention indices were measured.

Work on an extract of the sponge *Axinella* sp. 2 led to the isolation of two known biologically active components, girolline and hymenialdisine. Some discrepancies in the experimental and published data on hymenialdisine were noted and a previously unreported type of biological activity was detected for girolline.

A new sterol sulphate was isolated from an extract of the sponge *Stylopus australis* and two derivatives were synthesised. The full assignment of the ^{1}H nmr spectrum of the sulphate was achieved. An extract of a related sponge, *Hymedesmia* sp. 1 was examined and an unusual amino acid found to be a major water soluble component of the sponge. The sterols of both *Stylopus australis* and *Hymedesmia* sp. 1, along with those of *Hymedesmia* sp. 2 were examined for comparative purposes.

A new furan fatty acid was isolated from an extract of the sponge *Hymeniacidon hauraki*. The isolation of the sterol corbisterol and the corresponding

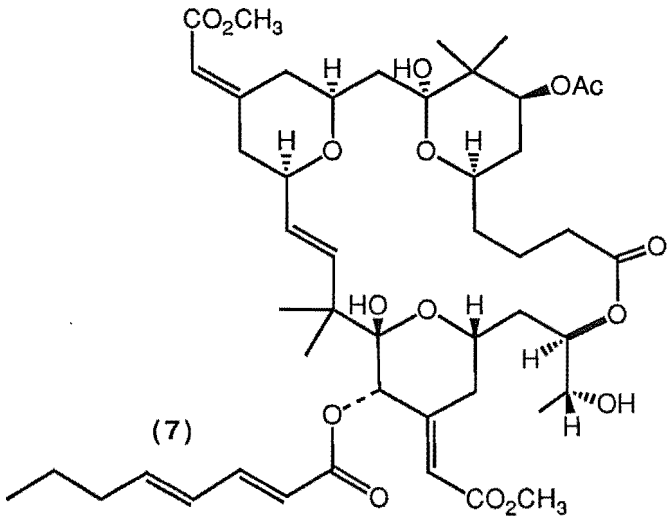
sterol peroxide from one *H. hauraki* sample, led to an examination of infraspecific sterol variation in this sponge species.

A screening procedure has been developed which examines the chromatographic behaviour of an extract coupled with its biological activity, to rapidly detect known biologically active compounds and to gain information on the best means of dealing with extracts containing unknown active components. A modification of this procedure is now used by the group to assess the suitability of an extract for further research.

CHAPTER TWO

2.1 COMPOUNDS ISOLATED FROM BRYOZOANS-A REVIEW

It is only within the last ten to fifteen years that researchers in marine natural products have begun to study the bryozoans, which are also called moss animals. A bryozoan is actually a sedentary colony of minute filter feeding individuals or zooids and these organisms are widespread throughout the marine environment[18]. They have proved to be worthy of attention as rich sources of biologically active compounds and/or compounds with novel structures. Their chemical components have been reviewed by Christophersen in 1985[18] and by Faulkner in his reviews in *Natural Product Reports*[19]. A more recent review, encompassing the information contained in these past reviews, while updating the groups of compounds isolated from bryozoans is presented here.



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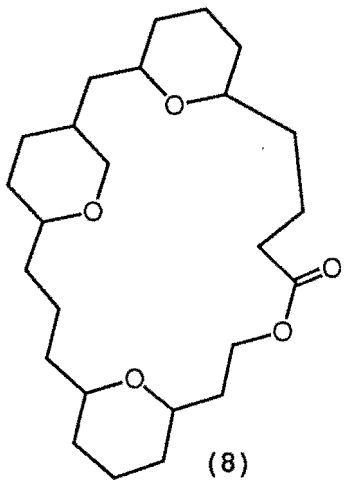
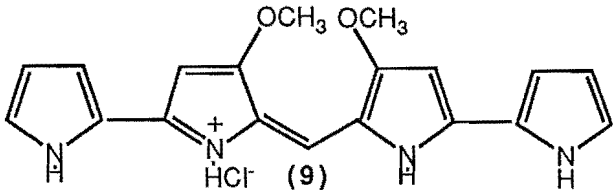


(8)

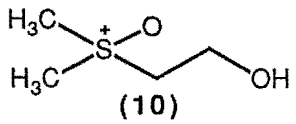
Of the compounds isolated thus far, the bryostatins are perhaps the most interesting, possessing both novel structures and high antineoplastic activities. The structure of the first of these, bryostatin 1 (**7**) was confirmed by single crystal X-ray crystallography[20] and to date nineteen related compounds, bryostatins 1-17 and bryostatins A and B have been reported[20-29]. All of these compounds were isolated from the marine bryozoan *Bugula neritina*, with the exception of bryostatin 8 which was isolated from the Gulf of Mexico bryozoan *Amathia convoluta*[24] and bryostatins A and B, isolated from the sponge *Lissodendoryx isodictyalis*[29]. It seems however that

in both these latter cases the samples had *Bugula neritina* attached and some symbiotic relationship between the species has been suggested[29]. All bryostatins possess the unusual 26-membered bryopyran ring system (**8**) and are typically present in the animal at an extremely low concentration, (~ 10^{-6}% by weight)[8]. These compounds are promising candidates for cancer chemotherapy on account of their very potent *in vivo* antitumour activity.

Another species of *Bugula, B. dentata* was shown to contain an antimicrobial blue pigment (**9**). This tetrapyrrole was previously isolated from a tunicate and a bacterium[30].

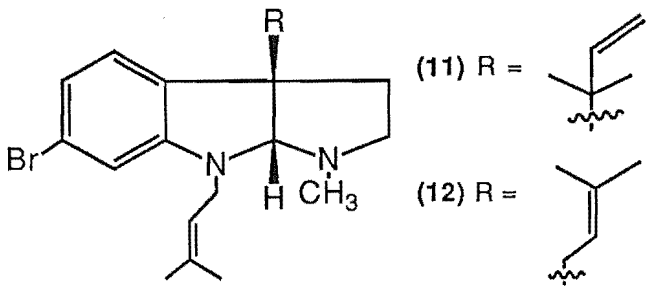


(**9**)



(**10**)

"Dogger Bank itch" is an allergic contact dermatitis, caused by contact with the bryozoan *Alcyonidium gelatinosum*. The compound responsible for this, (2-hydroxyethyl)dimethylsulphoxonium ion (**10**), has been isolated from the bryozoan by a combination of gel permeation and ion exchange chromatography, followed by precipitation as an iodo complex. The concentration of the sulphoxonium salt is estimated at 5 ppm of animal wet weight[31]. Synthesis of this compound was achieved by base-catalysed condensation of formaldehyde with trimethylsulphoxonium chloride[31].



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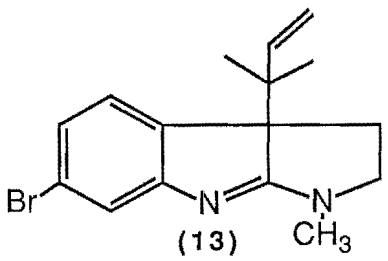
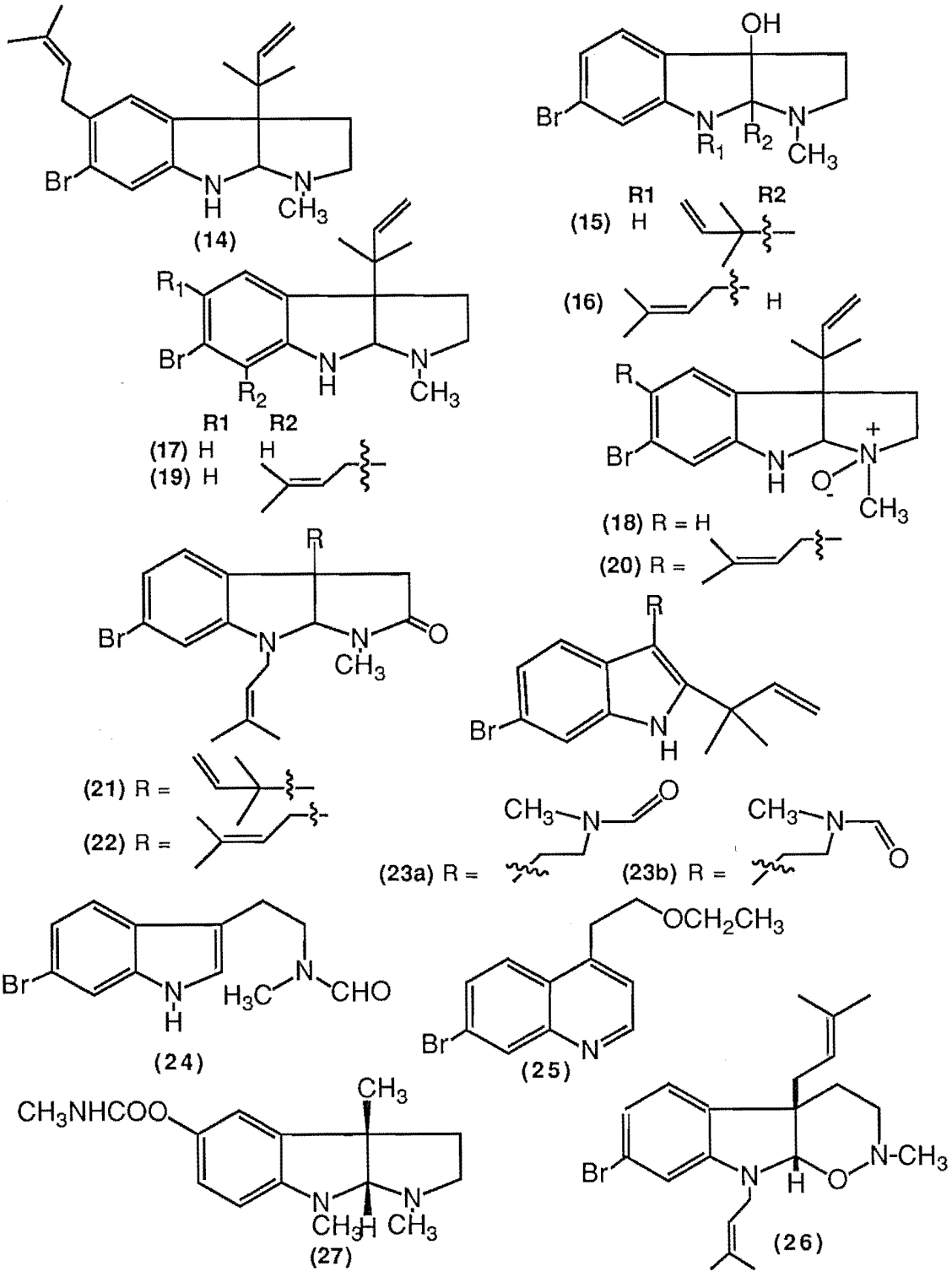
(**12**) R =



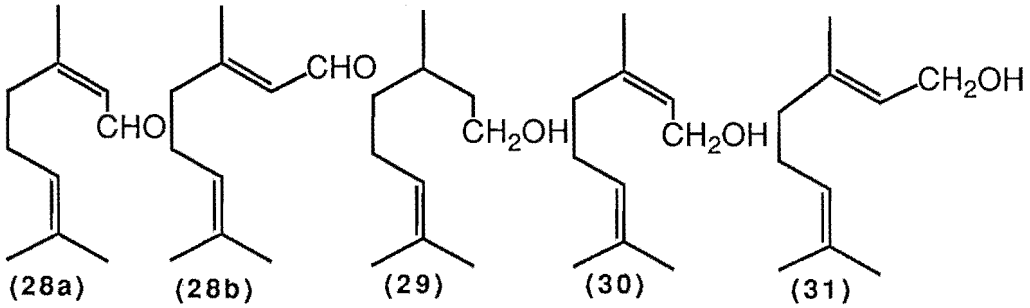
(**13**)

A series of brominated alkaloids has been isolated from the marine bryozoan *Flustra foliacea*. These are flustramines A-D (**11-14**)[32-34], flustraminols A (**15**) and B (**16**)[33], dihydroflustramine C (**17**)[33,35] and its N-oxide (**18**)[34], isoflustramine D (**19**) and flustramine D N-oxide (**20**)[34], flustramides A (**21**)[36] and B (**22**)[37], flustrabromine (**23**)[38], 6-bromo-N-formyl-N-methyltryptamine (**24**)[36], a bromoquinoline, 7-bromo-4(-2-ethoxyethyl) quinoline (**25**)[39] and flustrarine B (**26**)[37]. All the structures are

All the structures are formally derived from 6-bromotryptamine[40], with most possessing the skeleton of the alkaloid physostigimine (**27**). Flustramines A and B exhibit muscle relaxant activity both *in vitro* and *in vivo*[41], while dihydroflustramine C exhibits strong antimicrobial activity[34,35]. The crude petroleum ether extract of *F. foliacea* shows strong inhibitory activity against influenza virus[18].

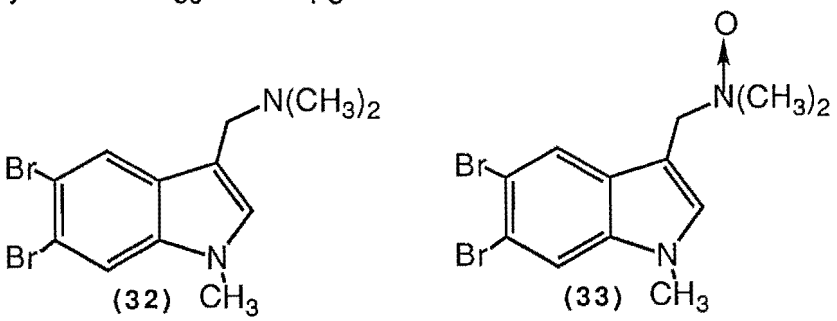


In earlier work on *F. foliacea*, the terpenoids *cis*- and *trans*- citral (**28a-b**), citronellol (**29**), nerol (**30**) and geraniol (**31**) were isolated[42]. This mixture displayed antifouling activity comparable to that of commercially available preparations[18].



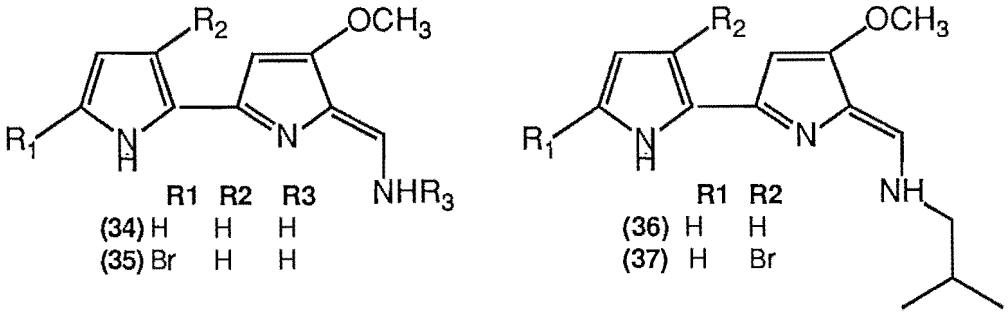
(28a) (28b) (29) (30) (31)

The bromo-alkaloids, 2,5,6-tribromo-N-methylgramine (**32**) and its N-oxide (**33**) were isolated from the subtropical marine bryozoan *Zoobotryon verticillatum*[43]. Both alkaloids were synthesised from 3-dimethylaminomethyl indole (gramine)[43]. 2,5,6-tribromo-N-methylgramine (**32**) inhibits cell division in the fertilized sea urchin egg assay with an ED_{50} of ~ 16 µg/ml.



(32) (33)

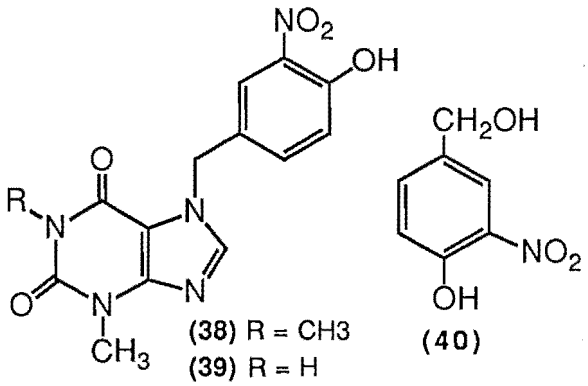
A series of bipyrroles, tambjamines A-D (**34-37**), were isolated from the bryozoan *Sessibugula translucens*, but are so called because they were originally found in nudibranchs of the genus *Tambje*, a predator of the bryozoan. These compounds are potent inhibitors of cell division in the fertilsed sea urchin egg assay and also exhibit antimicrobial properties[44].



	R1	R2	R3
(34)	H	H	H
(35)	Br	H	H

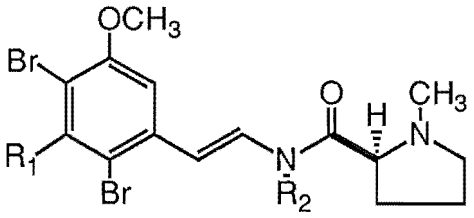
	R1	R2
(36)	H	H
(37)	H	Br

Phidolopin (**38**), a purine derivative, was isolated from the bryozoan *Phidolopora pacifica* and the structure solved by X-ray crystallography[45]. It is both strongly antifungal and antialgal *in vitro* and a total synthesis has been accomplished[46]. Two further nitrophenols, desmethylphidolopin (**39**) and 3-nitro-4-hydroxybenzyl alcohol (**40**) were subsequently isolated from the same species and desmethylphidolopin was synthesised[47].



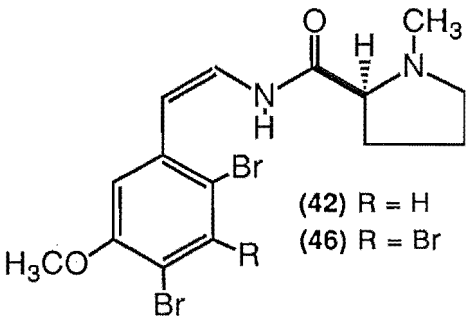
(**38**) R = CH3
(**39**) R = H

(**40**)

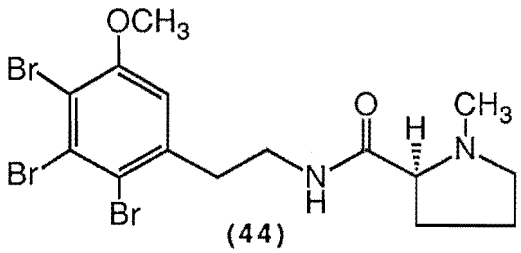


	R1	R2
(**41**)	H	H
(**43**)	Br	CH_3
(**45**)	Br	H

Another group of brominated alkaloids, the amathamides, have been isolated from the Tasmanian bryozoan *Amathia wilsoni*. Six amathamides, A-F (**41-46**)[48,49] were reported and variations in alkaloid content between samples from different collection sites were noted[49].

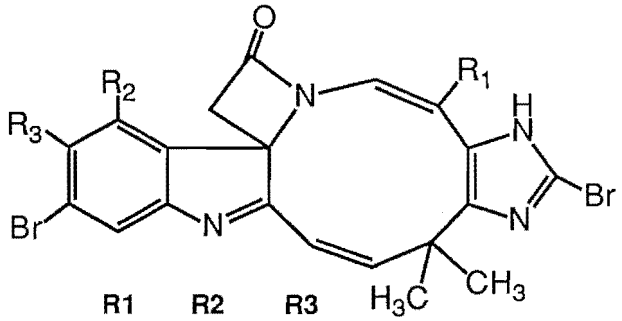


(**42**) R = H
(**46**) R = Br

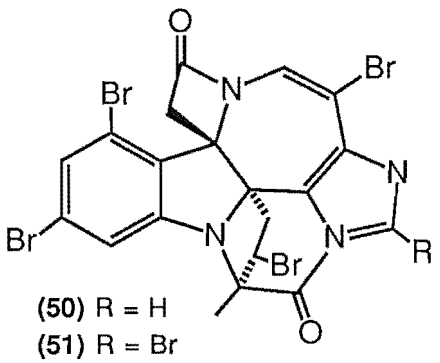


(**44**)

A bryozoan of the same family (Flustridae) as *F. foliacea*, *Chartella papyracea*, is the source of a series of unusual halogenated β-lactam alkaloids. Chartellines A-C (**47-49**)[50,51] and chartellamides A (**50**) and B (**51**)[52] have been isolated, along with methoxydechlorochartelline A (**52**)[51]. The structure of chartelline A was solved by X-ray crystallography. Methoxydechlorochartelline A is thought to be an artefact of the isolation procedure and was synthesised from chartelline A.

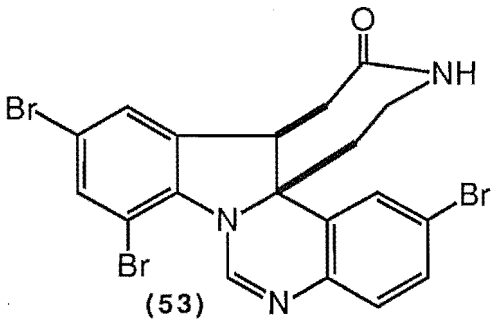


	R1	R2	R3
(47)	Cl	Br	Br
(48)	H	Cl	Br
(49)	Cl	H	H
(52)	OCH_3	Br	Br

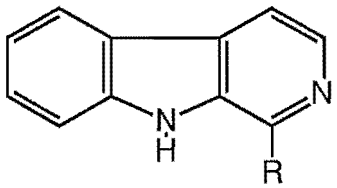


(50) R = H
(51) R = Br

An unusual tribrominated alkaloid, hinckdentine A (**53**) was isolated from the marine bryozoan *Hincksinoflustra denticulata*, and the structure solved by X-ray crystallography[53].



(53)



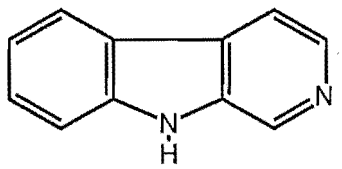
(54) R = $CH(OH)CH_3$
(55) R = CH_3
(56) R = CH_2CH_3
(57) R = $CH{=}CH_2$

The Tasmanian bryozoan, *Costaticella hastata* was the source of a group of β-carboline alkaloids, including the new compound (S)-1-(1-hydroxyethyl)-β-carboline (**54**), 1-methyl-β-carboline, (harman) (**55**), 1-ethyl-β-carboline (**56**) and 1-vinyl-β-carboline, (pavettine) (**57**)[54].

2.2 STUDIES OF THE BRYOZOAN, *CRIBRICELLINA CRIBRARIA.*

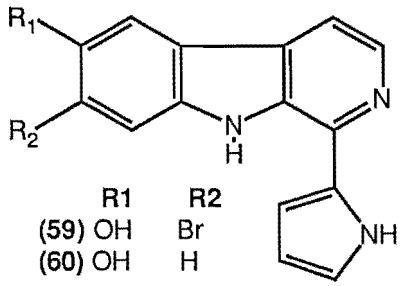
INTRODUCTION

Although the simple β-carboline alkaloids are well known from terrestrial plants[55], reports of their occurrence in the marine environment are scarce. They were first reported from a marine animal when Inoue *et al.* isolated harman (**55**) and β-carboline (norharman) (**58**) from the dinoflagellate, *Noctiluca miliaris*[56].

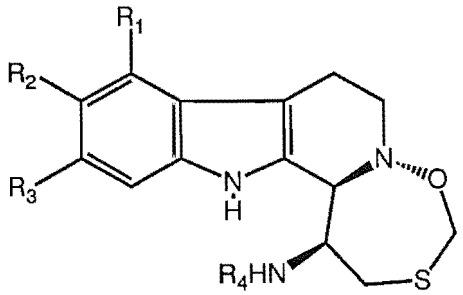


(58)

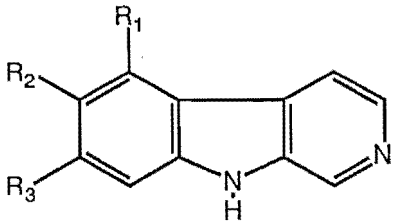
Later, Rinehart *et al.* isolated the eudistomins (**59-73**), a series of potent antiviral compounds, from the Caribbean tunicate *Eudistoma olivaceum*[57] and compounds of this type were subsequently isolated from a New Zealand ascidian *Ritterella sigillinoides*[58]. Three other eudistomins (**74-76**) have since been isolated from *Eudistoma olivaceum*[59].



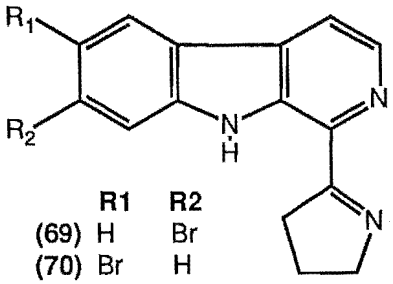
	R1	R2
(59)	OH	Br
(60)	OH	H



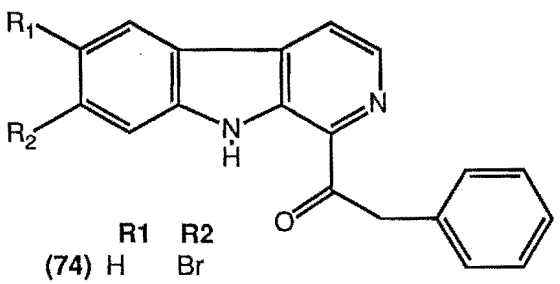
	R1	R2	R3	R4
(61)	H	OH	Br	H
(62)	Br	OH	H	H
(63)	H	H	Br	H
(64)	H	Br	H	H



	R1	R2	R3
(65)	Br	OH	H
(66)	H	OH	Br
(67)	H	Br	H
(68)	H	H	Br

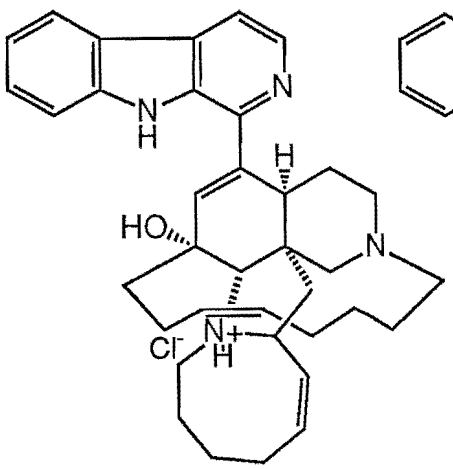


	R1	R2
(69)	H	Br
(70)	Br	H
(71)	H	H
(72)	OH	Br
(73)	OH	H

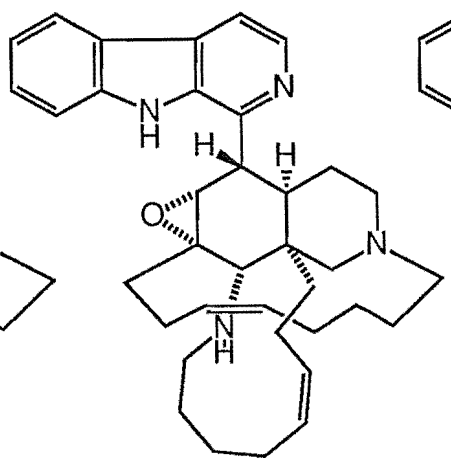


	R1	R2
(74)	H	Br
(75)	Br	H
(76)	H	H

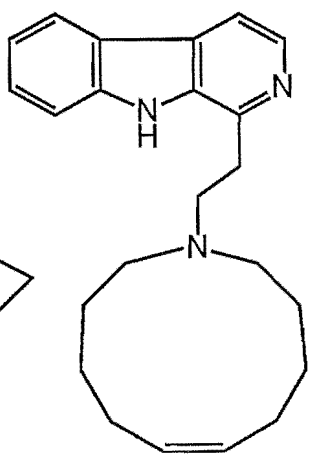
Jefford *et al.* isolated the compounds, manzamines A, B and C, (**77-79**) from the sponge *Haliclona* sp.[60,61]. The IC_{50} of manzamine A against P388 was determined to be 0.07 μg/ml, while those of manzamines B and C were determined to be 6 and 3 μg/ml respectively. At around the same time, Nakamura *et al.* reported the isolation of two β-carboline alkaloids from the Okinawan sponge, *Pellina* sp., terming them keramamines A and B[62]. Both exhibited antimicrobial activity with minimum inhibitory concentrations against *Staphylococcus aureus* of 6.3 and 25 μg/ml respectively. Keramamine A is identical in structure to manzamine A. Further manzamines, E (**80**) and F (**81**) were isolated from an Okinawan sponge, *Xestospongia* sp., both with an IC_{50} of 5 μg/ml[63]. Through direct comparison of manzamine F and keramamine B by tlc, uv and ir spectroscopy, both compounds were shown to be identical and the structure of keramamine B revised[63]. It was postulated that the isolation of this class of compounds from three different sponge genera, implies that the producer of these is a symbiotic microorganism.



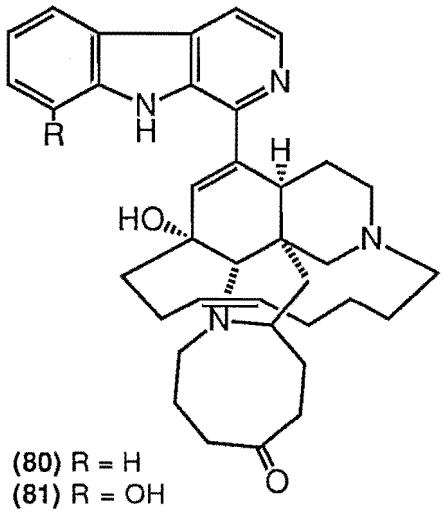
(77)



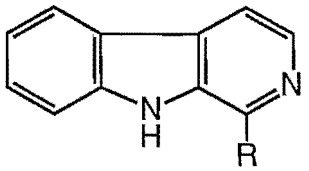
(78)



(79)

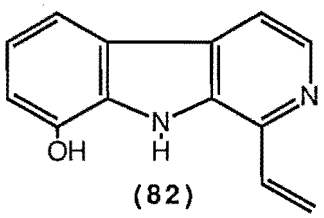


(80) R = H
(81) R = OH



(54) R = $CH(OH)CH_3$
(55) R = CH_3
(56) R = CH_2CH_3
(57) R = $CH{=}CH_2$

As mentioned above, simple β-carboline alkaloids (**54-57**) have been isolated from a marine bryozoan *Costaticella hastata* from Southern Tasmania[54]. This genus is considered to be analogous to *Cribricellina*[64]. No mention of any biological activity of the extract or of the isolated compounds was made.



(82)

A study of the marine bryozoan, *Cribricellina cribraria* was undertaken, as the crude extract was strongly cytotoxic against P388 and exhibited strong cytotoxicity on the antiviral/cytotoxicity assay. This led to the isolation of a new β-carboline alkaloid, 1-ethenyl-9H-pyrido[3,4-b]indol-8-ol (1-vinyl-8-hydroxy-β-carboline) (**82**), as the major cytotoxic component of the extract, along with a number of other minor β-carboline alkaloids. Several derivatives of compound (**82**) were also prepared. A novel β-carboline alkaloid with a 4-methylsulphone substituent was isolated as a minor component of the extract. Homarine was isolated as the main water soluble component, while cholesterol, and cholest-4-en-3-one were the major components of the sterol mixture isolated from the bryozoan.

ISOLATION OF 1-VINYL-8-HYDROXY-β-CARBOLINE (**82**).

A methanol/toluene extract of the orange bryozoan *Cribricellina cribraria*, showed strong P388 activity and cytotoxicity against a BSC cell line. Flash column chromatography on C18 reverse phase material led to the activity being concentrated in two fractions from the column. Recombination of these and further reverse phase column chromatography concentrated the activity into one fraction, from which further reverse phase column chromatography led to the isolation of the new compound 1-vinyl-8-hydroxy-β-carboline (**82**) as a yellow oil and the major cytotoxic component of the extract. For the isolation scheme used refer to Figure A.1 in the Appendix.

The structure of (**82**) was determined from the results of 1- and 2-dimensional nmr spectroscopic experiments. The ^{1}H nmr spectrum of (**82**) in CD_3OD (Figure 2.1) showed signals for 8 protons, each in a different chemical environment. The ^{1}H nmr data for (**82**) are summarised in Table 2.1. The ^{13}C nmr spectrum of (**82**) (Table 2.2) revealed the presence of twelve carbon resonances, of which seven were protonated carbons. Results of a COSY experiment showed three distinct proton spin systems, and a HETCOR experiment showed that two of the protons were attached to the same carbon. These results allowed the assembly of the structural fragments shown in Figure 2.2.

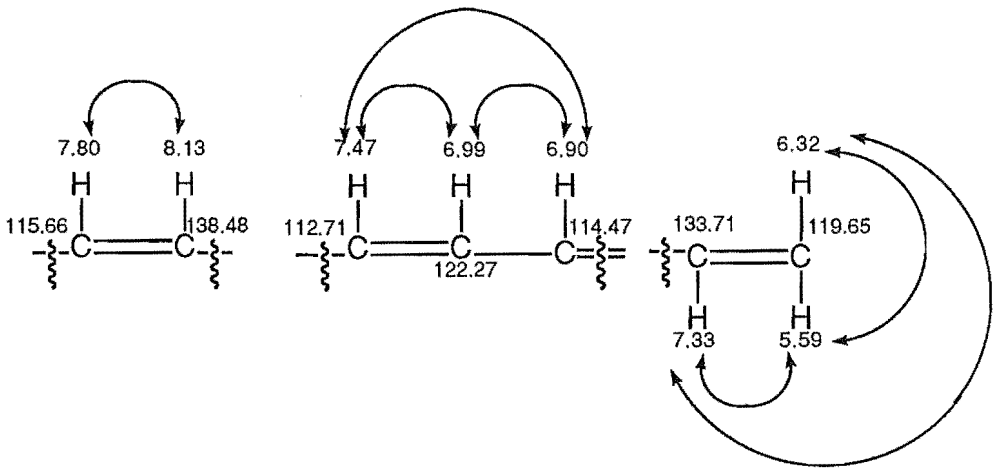


Figure 2.2: Structural fragments of compound (**82**), assembled from the results of COSY and HETCOR nmr experiments.

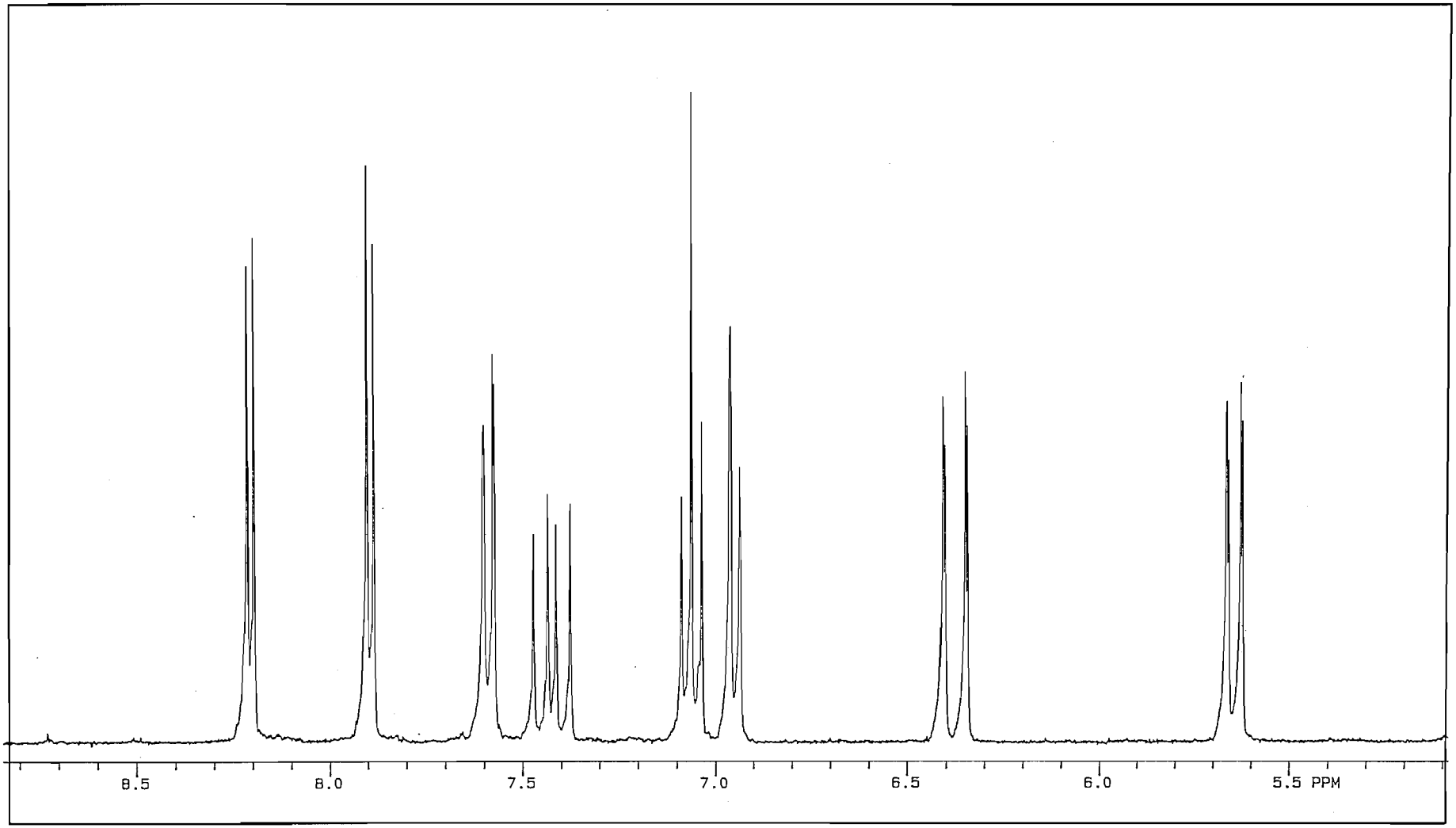


Figure 2.1: The ^{1}H nmr spectrum of 1-vinyl-8-hydroxy-β-carboline (**82**) in CD_3OD.

Table 2.1: ^1H nmr data for compounds (**57**), (**82**) and (**88-91**).

POSITION	(57)[a]	(82)[b]	(88)[a]	(89)[b]	(90)[b]	(91)[b]
3	8.47 d (5.2)	8.13 d (5.3)	8.44 d (5.2)	8.14 d (5.5)	8.30 d (5.3)	8.08 d (5.5)
4	7.87 d (5.2)	7.80 d (5.3)	7.84 d (5.2)	7.85 d (5.5)	8.01 d (5.3)	7.80 d (5.5)
5	8.13 dd (7.9, 0.9)	7.47 dd (7.7, 0.9)	7.70 d (7.9)	7.61 d (7.8)	8.07 dd (7.5, 1.8)	7.63 d (7.6)
6	7.29 m	6.99 t (7.7)	7.22 t (7.9)	7.06 t (7.8)	7.25 t (7.5)	7.10 t (7.6)
7	7.52 m	6.90 dd (7.7, 0.9)	7.00 d (7.9)	6.94 d (7.8)	7.31 dd (7.5, 1.8)	6.99 d (7.6)
8	7.52 m					
9	8.9 br s	8.5 br s[a]	8.5 br s		8.5 br s[a]	
1'	7.22 dd (17.5, 11.1)	7.33 dd (17.5, 11.1)	7.22 dd (17.5, 11.1)	3.19 q (7.6)	7.47 dd (17.2, 11.0)	3.11 q (7.6)
2'a	6.39 dd (17.5, 1.3)	6.32 dd (17.5, 1.4)	6.41 dd (17.5, 1.4)	1.41 t (7.6)	6.43 dd (17.2, 1.8)	1.31 t (7.6)
2'b	5.67 dd (11.1, 1.3)	5.59 dd (11.1, 1.4)	5.71 (11.1, 1.4)		5.66 dd (11.0, 1.8)	
8'			4.03 s		2.45 s	3.97 s

[a] Values in ppm for $CDCl_3$ solutions.
[b] Values in ppm for CD_3OD solutions.
() coupling constants in Hz.

Table 2.2: ^{13}Cnmr data for compounds (**57**), (**82**) and (**88-91**).

CARBON	(57)[a]	(82)[b]	(88)[a]	(89)[b]	(90)[b]	(91)[b]
1	139.84	141.23	140.12	149.11	141.56	148.32
3	139.17	138.48	139.19	138.06	139.48	138.23
4	114.07	115.66	114.32	114.48	115.82	114.48*
4a	129.96	132.27	130.11	130.93	132.48	126.25
4b	coincident	124.12	122.77	124.65	122.40	120.62
5	121.75	112.71	113.86	113.70	121.45	114.91*
6	120.32	122.27	120.78	121.78	120.63	121.68
7	128.54	114.47	107.95	113.46	122.54	109.25
8	111.59	147.15	146.20	145.69	147.09	144.83
8a	140.37	133.71	130.85	132.97	139.04	134.50
8b	133.63	135.24	133.24	135.64	136.45	136.91
1'	132.97	133.71	132.98	28.13	132.64	28.13
2'	119.12	119.65	119.09	14.05	120.08	14.12
8-OCH_3			55.62			56.38
8-OAc					164.28 21.26	

[a] Values in ppm for $CDCl_3$ solutions.
[b] Values in ppm for CD_3OD solutions.
* Values in vertical columns may be interchanged.

The chemical shift data, together with the results of an XCORFE experiment suggested a β-carboline nucleus with a vinyl substituent at C1 and a substituent, X, at either C5 or C8. Selected XCORFE nmr correlations are shown in Figure 2.3.

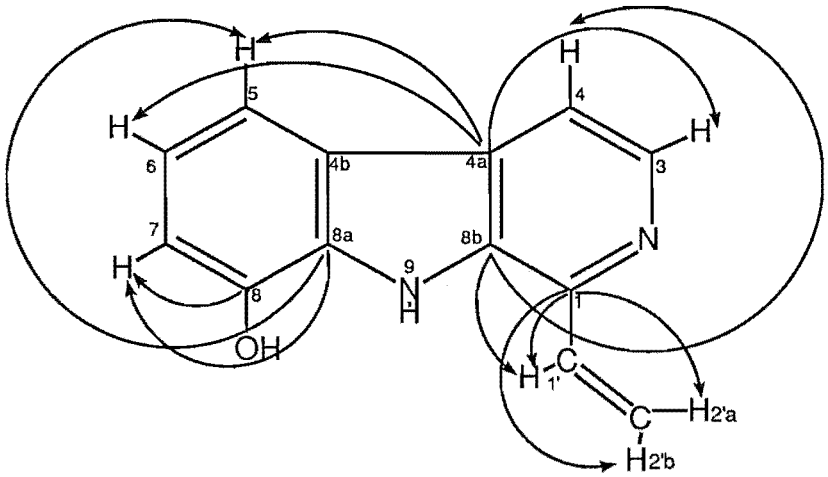


Figure 2.3: Selected XCORFE correlations for compound (**82**).

The position of substitution was determined by a series of nOe experiments. Irradiation of the H4 resonance led to enhancement of the H3 signal and the H5 signal, while the reverse irradiation of the H5 resonance led to enhancement of the H6 and H4 resonances (Figure 2.4). This determined the site of substitution as C8. The two dimensional nmr spectroscopic data and nOe enhancements observed for compound (**82**) are listed in Table 2.3.

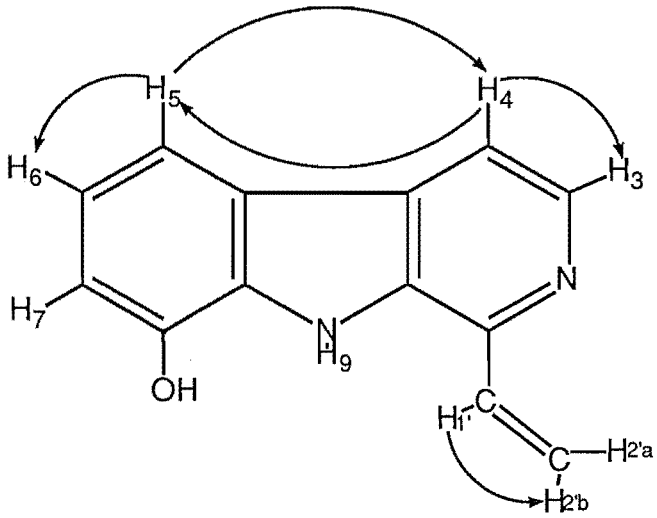
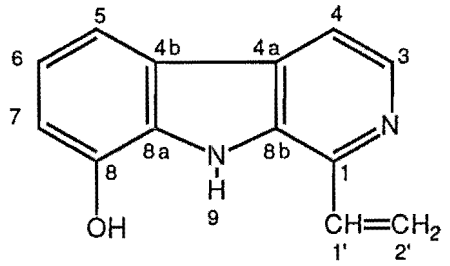


Figure 2.4: nOe enhancements observed for compound (**82**).

Table 2.3: COSY, HETCOR and XCORFE correlations and nOe enhancements observed for compound (**82**).

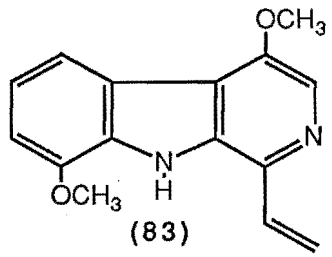


COSY	H3	<->	H4	HETCOR	H3	<->	$C_{138.48}$
	H5	<->	H6		H4	<->	$C_{115.66}$
	H5	<->	H7		H5	<->	$C_{112.71}$
	H6	<->	H7		H6	<->	$C_{122.27}$
	H1'	<->	H2'a		H7	<->	$C_{114.47}$
	H1'	<->	H2'b		H1'	<->	$C_{133.71}$
	H2'a	<->	H2'b		H2'a	<->	$C_{119.65}$
					H2'b	<->	$C_{119.65}$

XCORFE	H3	<->	C4		H7	<->	C5
		<->	C4a			<->	C8
	H4	<->	C3			<->	C8a
		<->	C8b		H1'	<->	C1
	H5	<->	C4a			<->	C4a
		<->	C7		H2'a	<->	C1
		<->	C8a			<->	C1'
	H6	<->	C4a		H2'b	<->	C1

nOe[a]	H4	<->	H3, H5
	H5	<->	H4, H6
	H6	<->	H5
	H1'	<->	H2'b

[a] nOe experiments were performed under non-spinning conditions.

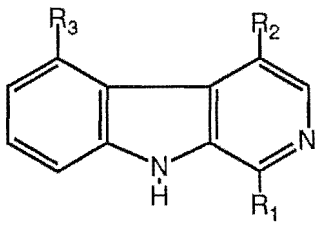


(83)

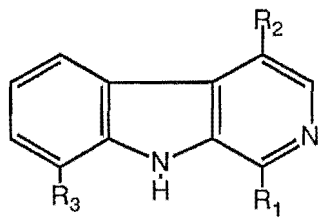
The presence of an oxygen substituent was suggested by comparison of ^{13}C nmr data with those of 1-vinyl-4,8-dimethoxy-β-carboline (**83**)[65]. Evidence for a hydroxyl substituent was the observation of a bathochromic shift in the uv spectrum with base from 369, 296, 244, 230 nm to 386, 287, 262, 240 nm. A hydroxyl stretching band in the ir spectrum of (**82**) provided further support for the presence of a phenol. The high resolution EI mass spectrum confirmed the proposed structure, with an $[M]^+$ of 210.0787, as appropriate for a molecular formula of $C_{13}H_{10}N_2O$.

METHYLATION OF (**82**)

To prove the nature of the oxygen substituent at C8 and to investigate the effects of structural modification on biological activity, methylation of (**82**) was carried out using diazomethane in ether. Methylation of compounds isolated from the tree, *Picrasma javanica*, originally identified as 1-vinyl-4-methoxy-5-hydroxy-β-carboline (**84**) and 1-ethyl-4-methoxy-5-hydroxy-β-carboline (**85**)[66], but later proven to be 1-vinyl-4-methoxy-8-hydroxy-β-carboline (**86**) and 1-ethyl-4-methoxy-8-hydroxy-β-carboline (**87**)[67], had resulted in a complex mixture of products[66]. The reaction mixture from the methylation of (**82**) in diazomethane was also a complex mixture. The major component of the mixture however was 1-ethenyl-8-methoxy-9H-pyrido-[3,4-b] indole (1-vinyl-8-methoxy-β-carboline) (**88**) and this was purified by reverse phase column chromatography.



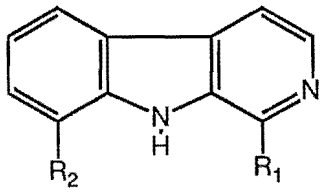
	R1	R2	R3
(84)	$CH=CH_2$	OCH_3	OH
(85)	CH_2CH_3	OCH_3	OH



	R1	R2	R3
(86)	$CH=CH_2$	OCH_3	OH
(87)	CH_2CH_3	OCH_3	OH

The ^1H and ^{13}C nmr spectra of (**88**) in $CDCl_3$ were consistent with the formation of a methoxyl at C8. In the ^1H nmr spectrum, a new singlet at 4.03 ppm was

assigned to the methoxyl protons, while appropriate changes in the chemical shifts of protons H5-7 were observed (Table 2.1). The ^{13}C nmr spectrum was mainly assigned through the use of 2-dimensional nmr techniques. A signal at 55.62 ppm was assigned to the methoxyl carbon while a HETCOR experiment was used to assign the rest of the protonated carbon signals. A **H**eteronuclear **M**ultiple-**B**ond **C**onnectivity or HMBC experiment, facilitated the assignment of the quaternary carbon signals and confirmed the assignments of the protonated carbon resonances. (See Work Described in Chapter Two, p. 156).



	R1	R2
(88)	$CH=CH_2$	OCH_3
(89)	CH_2CH_3	OH

HYDROGENATION OF (82)

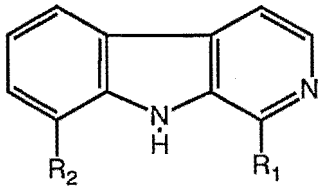
The dihydro derivative of (82), 1-ethyl-9H-pyrido-[3,4-b] indol-8-ol (1-ethyl-8-hydroxy-β-carboline) (89) was prepared by hydrogenation over platinum dioxide (Adam's catalyst). The ^{1}H and ^{13}C nmr spectra of (89) in CD_3OD were consistent with the formation of an ethyl group at C1. In the ^{1}H nmr spectrum, the vinyl proton signals were absent and replaced by a methylene quartet at 3.19 ppm and a methyl triplet at 1.41 ppm (Table 2.1). In the ^{13}C nmr spectrum, new signals at 28.13 and 14.05 ppm replaced the vinyl signals (Table 2.2). Full assignment of the ^{13}C nmr spectrum was again achieved with the aid of the results of an HMBC nmr experiment. (See Work Described in Chapter Two, p. 157).

ACETYLATION OF (82)

The 8-acetoxy derivative of (82), 1-ethenyl-8-acetoxy-9H-pyrido-[3,4-b] indole (1-vinyl-8-acetoxy-β-carboline) (90), was prepared by acetylation of (82) in pyridine/acetic anhydride.

The ^{1}H and ^{13}C nmr spectra of (90) were consistent with the formation of a monoacetate at position 8. A singlet in the ^{1}H nmr spectrum of (90) resonating at 2.45 ppm was assigned to the methyl protons of the acetate group, while downfield shifts were observed for the H5, H6 and H7 resonances (Table 2.1). Overlap of the H5 and H7 resonances in both $CDCl_3$ and CD_3OD, made unambiguous assignment of these signals impossible. In the ^{13}C nmr spectrum of (90), new signals at 164.28 and 21.26 ppm were assigned to the acetate carbons (Table 2.2). Again overlap of signals in $CDCl_3$ was a problem, but a HETCOR spectrum of (90), obtained in

CD_3OD, facilitated unambiguous assignment of the protonated carbon signals. (See Work Described in Chapter Two, p. 157).



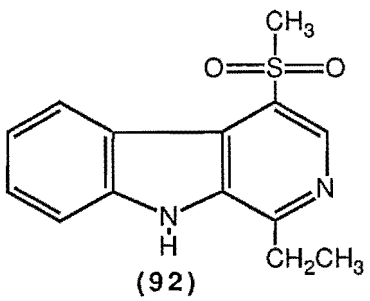
	R1	R2
(90)	$CH{=}CH_2$	OAc
(91)	CH_2CH_3	OCH_3

HYDROGENATION OF (**88**)

The dihydro derivative of (**88**), 1-ethyl-8-methoxy-9H-pyrido-[3,4-b] indole (1-ethyl-8-methoxy-β-carboline) (**91**), was prepared by hydrogenation over platinum dioxide in order that its biological activity could be determined (see below). The ^1H and ^{13}C nmr spectra of (**91**) in CD_3OD (Table 2.1) were consistent with the formation of an ethyl group at C1. The carbon signals were assigned by comparison with the ^{13}C nmr data for compounds (**88**) and (**89**). The structure of (**91**) was substantiated by comparison of the ^1H nmr and mass spectra with literature data[68].

ISOLATION OF 1-ETHYL-4-METHYL-SULPHONE-β-CARBOLINE (**92**)

During the course of isolation of (**82**), the presence of another compound, fluorescent under ultraviolet light and of similar Rf to (**82**) on tlc, was noted. A ^1H nmr spectrum of the appropriate fraction revealed that this was also a β-carboline alkaloid. Semi-preparative reverse phase hplc of fractions shown to contain this component by tlc and ^1H nmr spectroscopy, led to the isolation of 4 mg of (**92**) as a pale green oil, which fluoresced blue-purple in alcoholic solution.



(**92**)

The structure of (**92**) was proved by a combination of nmr spectroscopy and mass spectrometry. The ^1H nmr spectrum of (**92**) in $CDCl_3$ (Figure 2.5), indicated the presence of a β-carboline nucleus, with substitutents at either C1 and C3 or C1 and C4. Results of a COSY experiment showed a multiplet at 7.38 ppm was coupled to a doublet at 8.78 ppm (8.0 Hz) and a multiplet at 7.61 ppm (8.2 Hz), with signals in the ratio 1:1:2.

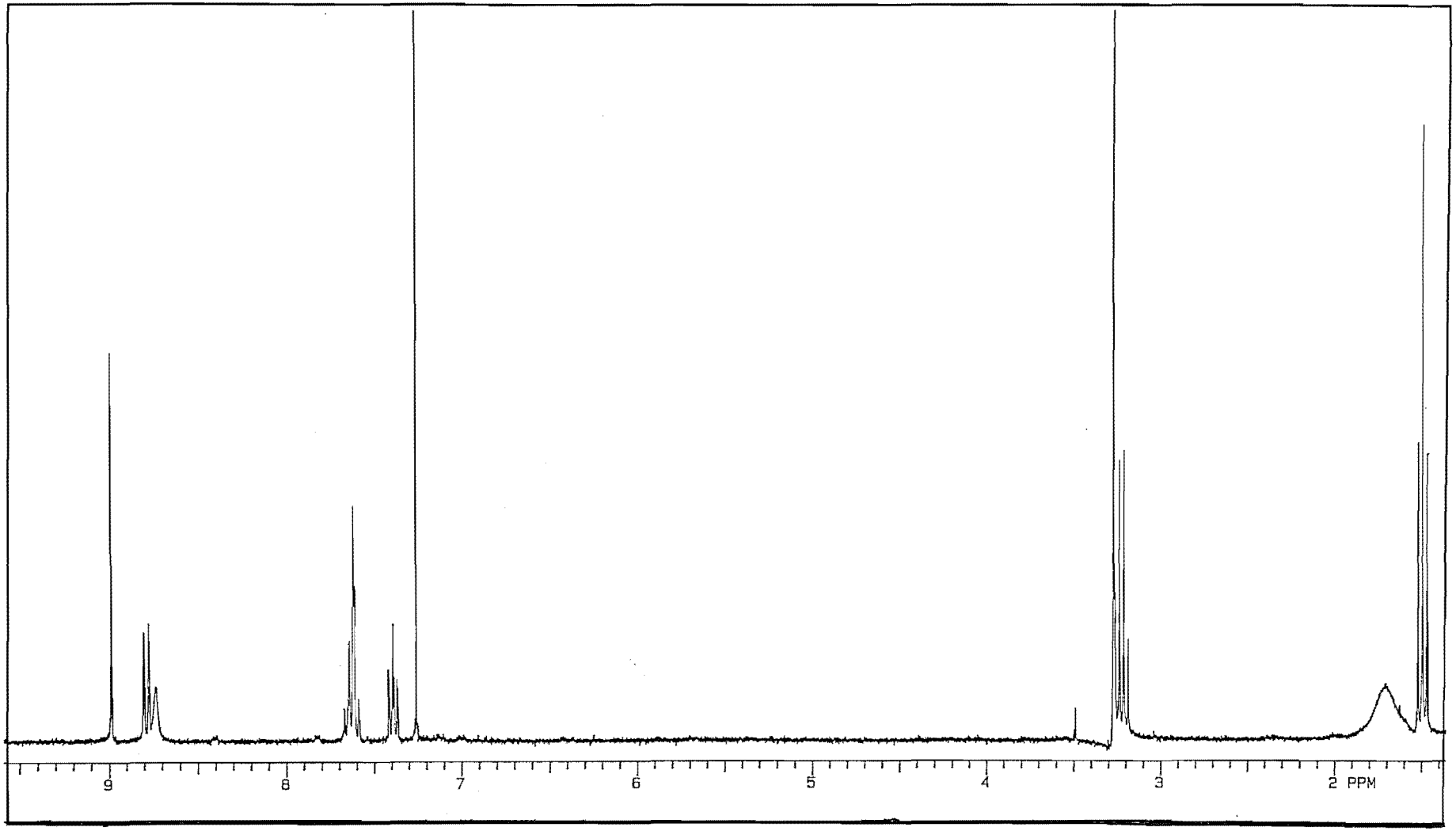


Figure 2.5: The ^{1}H nmr spectrum of 1-ethyl-4-methyl sulphone-β-carboline (**92**) in $CDCl_3$.

These data were consistent with an unsubstituted phenyl ring of a β–carboline nucleus, although the H5 signal was shifted downfield from the expected 8.1 ppm[69] to 8.78 ppm. Other features of the ^1H nmr spectrum were a one proton singlet at 8.98 ppm, a three proton singlet at 3.27 ppm, a three proton triplet and a two proton quartet assigned to an ethyl substituent and a broad one proton singlet at 8.7 ppm assigned to the indolic proton (Table 2.4).

The ^{13}C nmr spectrum of (**92**) in $CDCl_3$ (Table 2.4) revealed eight protonated carbon signals and six quaternary signals. The protonated carbon signals were assigned with the aid of a HETCOR experiment and the quaternary signals with the aid of an HMBC nmr experiment (Table 2.4).

A high resolution EI mass spectrum of (**92**) showed an $[M]^{+\bullet}$ ion of 274.0737 with an $[M+2]^{+\bullet}$ peak of the appropriate intensity for one sulphur atom. This data was suggestive of the molecular formula $C_{14}H_{14}N_2O_2S$. A DCI mass spectrum obtained using methane as reagent gas showed an $[MH]^+$ ion at 275, while that obtained using ND_3 as reagent gas showed a peak at 277 $[M-(H+D)D]^+$, confirming the presence of one exchangeable proton. The substitution pattern of the β-carboline nucleus was confirmed by a series of nOe experiments. Irradiation of the H9 signal led to enhancement of the H8 and H1' signals, confirming the presence of an ethyl substituent at C1. As no enhancement of the 8.98 singlet was observed on irradiation of the H5 resonance, it was considered that this signal arose from a proton attached to C3 and therefore that the site of substitution was C4. This left a fragment CH_3O_2S to be accounted for from the molecular formula which must be the C4 substituent. Fragmentations in the EI mass spectrum confirmed this grouping of atoms, with ions at 246 $[M-CH_2-CH_2]^{+\bullet}$, 194 $[M-H-CH_3SO_2]^{+\bullet}$ and 167 $[M-CH_3SO_2-CH_2CH_3]^+$. The singlet at 3.27 ppm in the ^1H nmr spectrum and a signal in the ^{13}C nmr spectrum at 43.24 ppm were consistent with a methyl group attached to a sulphur atom, in turn attached to one or more oxygen atoms. When the nOe experiments had been performed using $CDCl_3$ as solvent, it was not possible to irradiate the S-methyl group resonance, as the ethyl methylene quartet signal was coincident. When C_6D_6 was used as solvent however, these signals were resolved and could be irradiated separately. Irradiation of the H5 signal produced enhancement of the S-methyl signal and vice versa (Table 2.4).

Table 2.4: Nmr spectroscopic data for compound (**92**).

POSITION	$\delta\,^1H^a$	$\delta\,^{13}C^a$
1		152.70
3	8.98 s	139.88
4		119.22
4a		123.84
4b		126.67
5	8.78 d (8.0)	125.86
6	7.38 m	121.67
7	7.61 m	129.77
8	7.61 m	111.87
8a		140.57
8b		133.71
9	8.7 br s	
1'	3.22 q (7.6)	27.54
2'	1.50 t (7.6)	12.22
4'	3.27 s	43.24

$^1J_{CH}$ correlations (HETCOR)[a]	H3 <-> $C_{139.88}$	H8 <-> $C_{111.87}$
	H5 <-> $C_{125.86}$	H1' <-> $C_{27.54}$
	H6 <-> $C_{121.67}$	H2' <-> $C_{12.22}$
	H7 <-> $C_{129.77}$	H4' <-> $C_{43.24}$
$^1J_{CH}$,$^2J_{CH}$,$^3J_{CH}$ and $^4J_{CH}$ correlations (HMBC)[a]	H3 <-> C1, C4b, C4a	H9 <-> C8a
	H5 <-> C8a	H1' <-> C1, C1'
	H6 <-> C8	H2' <-> C1, C4a, C8b, C2'
	H7 <->C8a	
	H8 <-> C8a	H4' <-> C4'
selected nOes[b]	H5 <-> H4', H6 (solvent obscured)	
	H1' <-> H2'	
	H4' <-> H5	

[a]For $CDCl_3$ solutions.
[b]For C_6D_6 solutions.
() coupling constants in Hz.

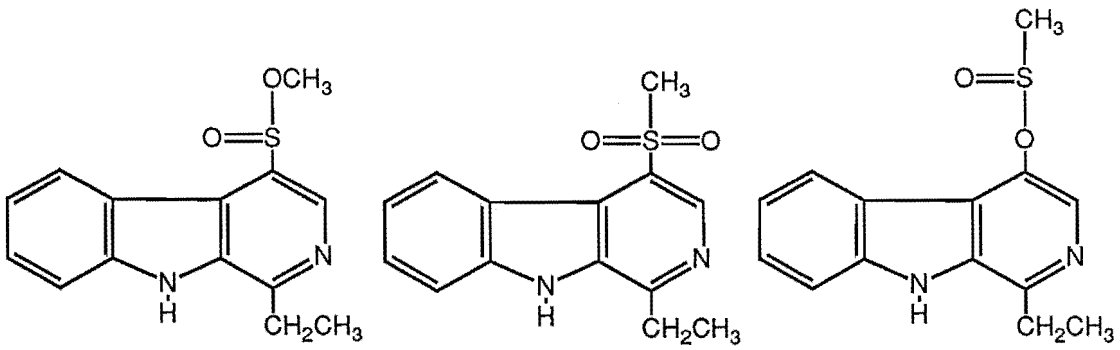


Figure 2.6: The three structural isomers possible for a 1-ethyl-β-carboline with a CH_3SO_2- substituent at position 4.

Three structural isomers, differing in the arrangement of atoms in the C4 substituent were possible (Figure 2.6). The isomer with a methoxyl group attached to the sulphur atom could be excluded on the basis of chemical shift data. Distinction of the two remaining possibilities was more difficult, with little or no difference expected in the chemical shifts of the methyl group, whether the compound was a methyl sulphone[70,71] or a methyl sulphinate ester[72,73]. The ^{13}C nmr chemical shift of C4 should be useful in distinguishing between these two possibilities and definitive assignment of this carbon was achieved from the results of an HMBC nmr experiment and a ^{13}C nmr experiment with a long pulse delay. One means of distinguishing between the two possibilities, using an HMBC nmr experiment, would be that in the case of a sulphone, one would expect to observe a three bond correlation from the H4' proton signal to the C4 signal and possibly also a four bond correlation from the H4' proton signal to the C3 signal and from the H3 proton signal to the carbon signal at the 4' position. For a sulphinate ester, no correlations would be observed as the correlations involved would range over five bonds. However, the only correlation observed from the H4' protons was the one bond correlation to the C4' resonance and correlations observed for the H3 proton were to quaternary signals which were assigned to other carbon atoms in the structure (Table 2.4).

This left a quaternary carbon signal at 119.22 ppm unassigned. Model compounds were used to gain an indication of where the C4 resonance would be expected to be observed for each of the two possible structures. 1-ethyl-4-methoxy-β-carboline (**93**) was used as a model for a compound with an oxygen attached directly to C4. For this compound, the C4 resonance was at 150.97 ppm and the C3 signal at 119.96 ppm[65], quite different from the observed 139.88 ppm shift for C3 of compound (**92**). Finding a suitable model for the sulphone possibility was more difficult. The difference in chemical shift of C3, C4 and C1 between toluene[74] and

methyl 4-tolyl sulphone[71] was compared with the difference in these resonances between 1-ethyl-β-carboline and compound (**92**). Using this model as a rough guide, for (**92**) one might expect a downfield shift of approximately 12 ppm in the C4 resonance, from the 112.91 ppm shift observed for 1-ethyl-β-carboline (see Table 3.2 below) and very little chemical shift difference for C3. A downfield shift of approximately 7 ppm would be expected for C1. This would mean that for (**92**), the C4 signal would be expected to be observed at approximately 125 ppm, although as the model is by no means ideal, some variation would be expected. The results of the model comparisons are summarised in Figure 2.7.

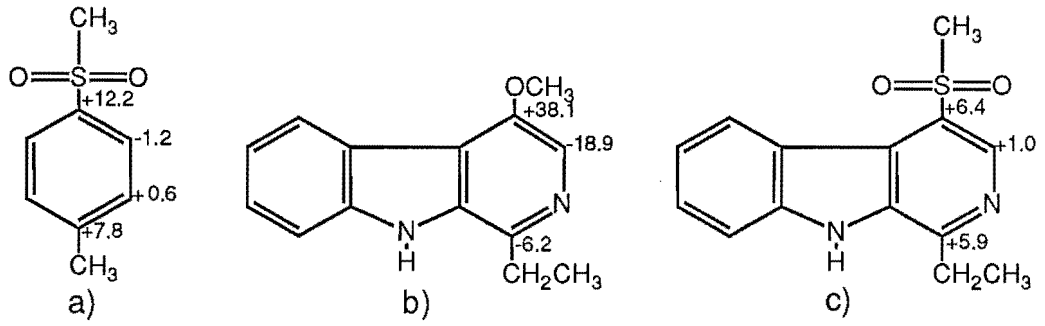


Figure 2.7: Differences in ^{13}C nmr chemical shifts in $CDCl_3$ on aromatic substitution
a) for toluene to methyl 4-tolyl sulphone
b) for 1-ethyl-β-carboline to 1-ethyl-4-methoxy-β-carboline (**93**)
c) for 1-ethyl-β-carboline to compound (**92**)

To check that the signal at 119.22 ppm was real, and that no other weak resonances were present, a ^{13}C nmr spectrum of compound (**92**) was obtained with a pulse delay of five seconds. No new signals were observed and that at 119.22 ppm was of similar intensity to the other assigned quaternary signals of the compound. This, combined with the better fit of the sulphone model compound to the observed data, supported the structure being assigned as the sulphone.

Mass spectrometry should also have been helpful in distinguishing between the two possible structures. For a sulphone, one would expect to see a fragment ion resulting from loss of a methyl group and a fragment ion resulting from complete loss of the sidechain. For a sulphinate ester, fragment ions corresponding to the loss of CH_2SO to form a phenol, and loss of the sidechain would be expected[72]. Under EI, DCI and DEI conditions at a variety of ionising voltages, the fragmentation observed was inconclusive, with compound (**92**) showing fragment ions corresponding to the loss of the 1-ethyl grouping, the entire C4 grouping and both of these substituents, but none corresponding to the loss of a methyl grouping or formation of a phenol.

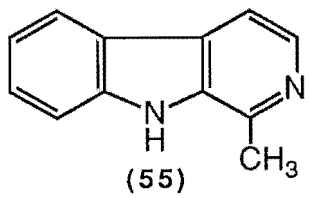
The ir spectrum of (**92**) had two strong bands at 1335 and 1160 cm^{-1}, typical of a sulphone[75], while in the uv spectrum of (**92**) in methanol, some bands were shifted to longer wavelength than in 1-ethyl-β-carboline, in keeping with the extended conjugation offered by the sulphone grouping.

Further evidence for a sulphone grouping rather than a sulphinate ester was given by the attempted acid hydrolysis of (**92**). Sulphinate esters are known to be very unstable and are easily hydrolysed under both acidic and basic conditions[72]. A sub-sample of (**92**) was dissolved in $CDCl_3$, D_2O/TFA added to form a bi-phasic system and the reaction (if any), monitored by ^1H nmr spectroscopy. After two days, the only differences in the spectrum were the disappearance of the exchangeable indolic proton and the appearance of an H-O-D peak at 4.7 ppm. The absence of any hydrolysis reaction again strongly favoured the sulphone structure.

This isolation of 1-ethyl-4-methyl sulphone-β-carboline, is the first report of a β-carboline alkaloid with a sulphur atom attached directly to the ring, either as a natural product or a synthetic compound.

ISOLATION OF HARMAN (**55**)

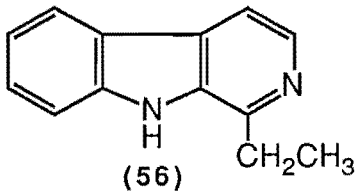
Tlc analysis of reverse phase column chromatography fractions less polar than those containing (**82**), revealed the presence of another compound which was also fluorescent under ultraviolet light. Further chromatography on C18 reverse phase material led to the isolation of harman (**55**). Harman was identified by mass spectrometry and by comparison of its ^1H and ^{13}C nmr spectra with literature data[76,65].



(**55**)

IDENTIFICATION OF 1-ETHYL-β-CARBOLINE (**56**)

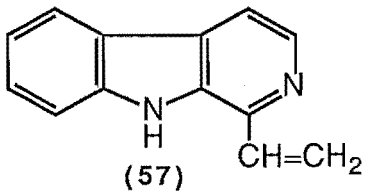
Fractions containing harman by tlc analysis also contained another component, fluorescent under ultraviolet light, at higher Rf on silica gel. A ^1H nmr spectrum of mixtures of this component and harman suggested that it was 1-ethyl-β-carboline and this was confirmed by mass spectrometry and tlc comparison with a synthetic sample, (see below).



(56)

ISOLATION OF PAVETTINE (**57**)

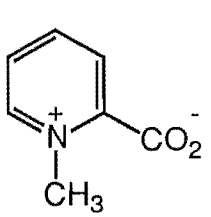
^{1}H nmr analysis of reverse phase chromatography fractions slightly more non-polar than those containing (**82**) revealed the presence of another component containing a vinyl substituent. A preparative tlc separation led to the isolation of less than 1 mg of a yellow-green oil, which was identified as pavettine (**57**), from its ^{1}H nmr and mass spectra[77] and by tlc comparison with an authentic sample.



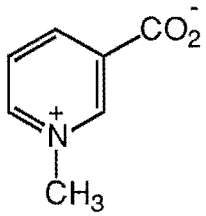
(57)

ISOLATION OF HOMARINE

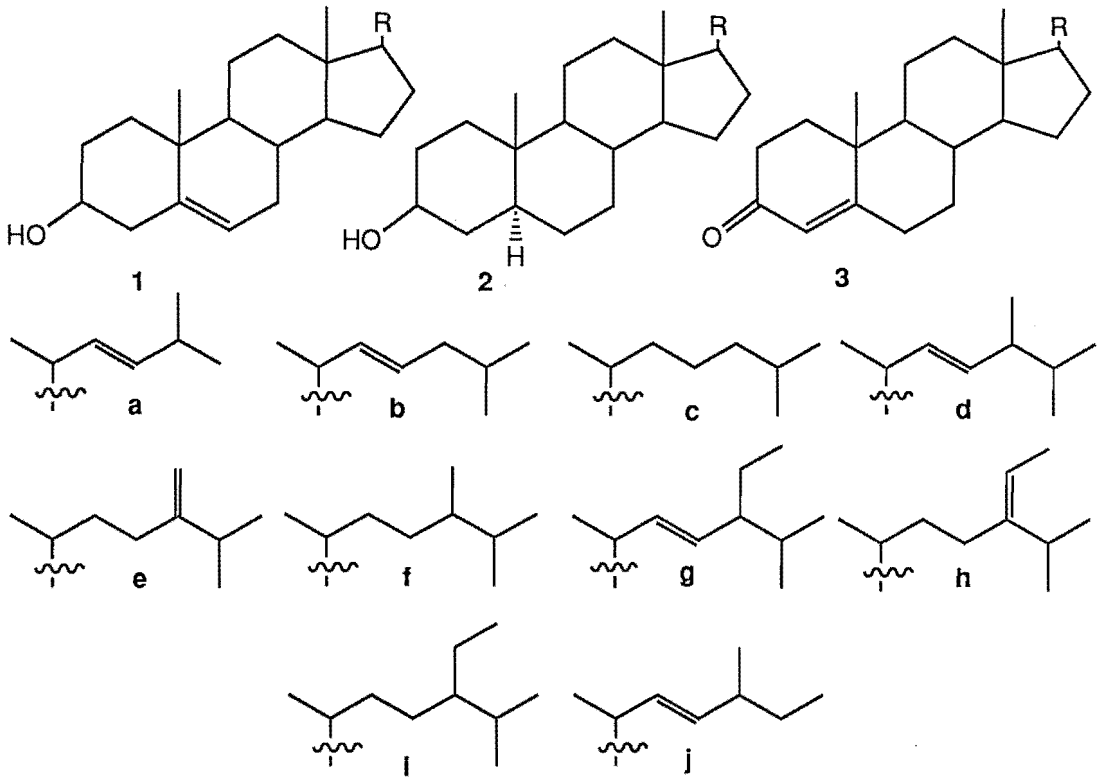
An early fraction from the initial reverse phase chromatography column on the extract contained a pale lemon precipitate, the ^{1}H nmr spectrum in D_2O of which was suggestive of a 2-substituted N-methyl pyridinium compound, reminiscent of homarine (**94**). This identification was confirmed by ^{13}C nmr spectroscopy[78]. This result is in accord with earlier findings, when homarine was identified as the major water soluble component of the bryozoan *Margaretta barbata*[79], and the results of Christophersen *et al.* who found homarine in addition to trigonelline (**95**) in each of four extracts of bryozoans investigated[80].



(94)



(95)



(96) 1a (100) 1d (104) 2c
(97) 1b (101) 1c (105) 1f
(98) 1j (102) 1h (106) 1l
(99) 1e (103) 1g (107) 3c

STEROLS

The sterols from the bryozoan were isolated by column chromatography on silica gel of a fraction from the initial reverse phase flash column on the extract. One fraction from the column contained a major component which was identified as the steroid, cholest-4-en-3-one (**107**) by comparison of its ^{13}C nmr spectra in $CDCl_3$ with literature data[81]. Cholesterol (**101**) was identified as the major component of the two subsequent fractions by comparison of the nmr spectroscopic data with literature values[81]. A sterol containing a 24-methylene group in the sidechain was also present in these fractions by ^{1}H and ^{13}C nmr spectroscopy, but in a lesser quantity. These three fractions were combined and sent to Professor Carl Djerassi for further analysis. The results of this analysis are summarised in Table 2.5 and confirm the presence of cholesterol and cholest-4-en-3-one as major components of the sterol mixture.

Table 2.5: The sterol composition of *Cribricellina cribraria.*

Sterols Present	Percentage composition of the sterol mixture of the *Cribricellina cribraria* sample.
(96)	3
(97)	9
(98)	+
(99)	4
(100)	18
(101)	28
(102)	+
(103)	4
(104)	13
(105)	+
(106)	6
(107)	10
unidentified	4

+ denotes a trace amount, <1%.

2.3 STUDIES OF THE BRYOZOAN, *MARGARETTA BARBATA*

PREVIOUS WORK ON *MARGARETTA BARBATA*

Earlier, preliminary work on a bryozoan of the same family (Micronellidae) as *Cribricellina cribraria*, *Margaretta barbata*, had been undertaken by Colin Barrow in 1987 as part of a Ph.D. thesis[79]. This work had been stimulated by the finding that the crude extract showed strong *in vivo* activity against P388. This earlier work had indicated that there were two biologically active components present in the extract, but with quite different polarities. After an initial reverse phase flash chromatography column on the crude extract, the more non-polar active material was concentrated into one fraction of 200 mg which eluted from the column with methanol. ^{1}H and ^{13}C nmr spectra of this fraction in $CDCl_3$ were obtained, with no clearly obvious features, apart from signals reminiscent of unsaturated long chain fatty acid material. Tlc

analysis of this fraction on silica gel revealed a number of spots which were fluorescent under uv light and well spread out on the plate. A preparative tlc separation on a sub-sample of this fraction on silica was attempted but this led to very low recovery of both mass and activity. Further work on this fraction was then abandoned.

ORGANIC SOLUBLE BIOLOGICAL ACTIVITY OF *MARGARETTA BARBATA*

In light of the biological activity associated with this bryozoan and the presence of the fluorescent spots, reminiscent of the β-carboline alkaloids isolated from *Cribricellina cribraria*, it was decided to investigate the appropriate fraction from *Margaretta barbata* more fully. The ^{1}H and ^{13}C nmr spectroscopy and assays were repeated to check the stability of the material. No significant differences were noted in the ^{1}H and ^{13}C nmr spectra or the tlc from those obtained previously. The fraction had an IC_{50} of 9100 ng/ml in the P388 assay and exhibited cytotoxicity against a BSC cell line of type C6.

Analytical reverse phase hplc was attempted and cuts taken and submitted for assay. Activity was spread throughout these cuts. A trial solvent partition between petroleum ether and ethanol/water (19:1), on a subsample of the material was attempted, to try and remove the fatty acid material. This was unsuccessful by both tlc analysis and biological assay, so the partitions were recombined.

Some of the sample had formed a dark brown solid, insoluble in methanol, dichloromethane or water, but a reverse phase flash chromatography column was run on the remaining 135 mg of sample. P388 assays on the column fractions showed significant activity in fractions 1-3 but the activity then dropped off markedly. Tlc of these fractions revealed the presence of the fluorescent spots. The fractions were recombined and a further reverse phase column run. Assays of these fractions revealed that the activity was primarily concentrated into one fraction from this column, which by tlc analysis, was not the fraction containing the fluorescent compounds. A ^{1}H nmr spectrum of the active fraction in $CDCl_3$ contained very broad and unresolved resonances and was suggestive of the presence of fatty acid type material. A trial methylation of a sub-sample of this fraction, using diazomethane in ether did not significantly alter the activity, so the whole sample was methylated in the same manner, in the hope that this would facilitate purification by converting the major contaminants to less polar compounds.

The methylated material was re-submitted to reverse phase hplc and cuts taken. Activity was concentrated into two fractions, the ^1H nmr spectra of which were similar to those previously acquired. The two fractions were recombined but some material was insoluble in $CDCl_3$ and remained on a filter when the nmr sample was prepared. This residue was dissolved in methanol and assayed separately from the $CDCl_3$ soluble fraction. P388 assay results showed that the activity was concentrated in the $CDCl_3$ soluble fraction which had been reduced in mass to 1.8 mg and which now had an IC_{50} of 270 ng/ml. A ^1H nmr spectrum of this fraction was obtained in $CDCl_3$ with a drop of TFA added in an attempt to sharpen the signals. This was not successful.

It was now obvious that the component responsible for the biological activity of the organic extract of the bryozoan was present at an extremely low level. Concentration of activity in the purification process was not accompanied by significant loss of mass and the remaining sample was still a complex mixture. Due to lack of material it was necessary to abandon work on this aspect of the active components from *Margaretta barbata*. The bioactivity-directed separation is summarised in Figure A.2 of the Appendix.

However, two known simple β-carboline alkaloids were recovered and characterised from the fraction from the reverse phase column on the organic soluble active component of *Margaretta barbata* that had been shown to contain components, fluorescent under uv light on tlc. The ^1H nmr spectrum of this fraction in $CDCl_3$ revealed aromatic signals, reminiscent of a β-carboline nucleus. Examination of the appropriate literature indicated that this was most likely, as some simple β-carboline alkaloids had been isolated from a Tasmanian bryozoan, *Costaticella hastata*, which is of the same family as *M. barbata*[54].

A singlet at 2.83 ppm in the ^1H nmr spectrum in $CDCl_3$ of this fraction indicated that the major component of the fraction was most likely harman (**55**), the major alkaloid isolated from *C. hastata*. Semi-preparative reverse phase hplc led to the isolation of 2 mg of an oil, the ^1H and ^{13}C nmr, uv and mass spectra of which, were identical to those reported for harman[76,65]. Tlc analysis revealed that this was the major component of the fluorescent compounds seen previously by tlc.

Another fraction from the reverse phase hplc run of < 1mg was shown by tlc to contain both harman and another component, fluorescent under uv light, at higher Rf on silica gel. No signals were visible when a ^1H nmr spectrum was attempted,

even after many transients. An EI mass spectrum of the fraction showed peaks corresponding to those of harman, and another component with an M^+ ion of 196. A peak at 168, corresponding to the loss of an ethyl grouping led to the component being tentatively identified as 1-ethyl-β-carboline. Comparison of the mass spectrum with that quoted for 1-ethyl-β-carboline confirmed this identification[69], as did tlc comparison with an authentic sample. This compound had also been isolated from *C. hastata* along with harman, and in a lesser amount, which makes its occurrence here not surprising.

CHAPTER THREE

3.1 FURTHER STUDIES OF β-CARBOLINE ALKALOIDS

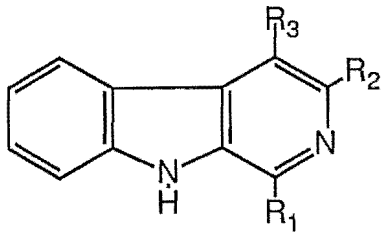
INTRODUCTION

The β-carboline alkaloids isolated from the bryozoan *Cribricellina cribraria* and their derivatives constitute a series of structurally related compounds, amenable to further studies. Other structurally related compounds could be prepared relatively simply to further extend the series. Several of these alkaloids were therefore synthesised and an nmr spectroscopic study and studies of the biological activity and structure-activity relationships within this series carried out.

The nmr spectroscopic study enabled the assignment of the ^{1}H and ^{13}C nmr spectra of the alkaloids, some of which had not been previously reported, while previously reported ^{13}C nmr spectroscopic assignments for some of the alkaloids were shown to be in error.

The main reason for synthesising further β-carboline alkaloids however, was to study their biological activities. A wide range of biological activities has been described for various β-carboline alkaloids. Many plants containing these alkaloids are used in traditional folk medicine to treat a variety of disorders. An example is the tropical forest tree of South America, *Picrasma crenata*, used in Southern Brazil to treat diabetes[68].

Other reported activities of these alkaloids include enzyme inhibition against cyclic adenosine monophosphate (cAMP) phosphodiesterase[82,83] and xanthine oxidase[84], antiviral[85,86] and antimicrobial[62,85]/antifungal[87] properties. Some have been used therapeutically in traditional medicine. For example, several of the alkaloids (**108-112**) have been used in the treatment of gout, urinary and renal calculi[84].

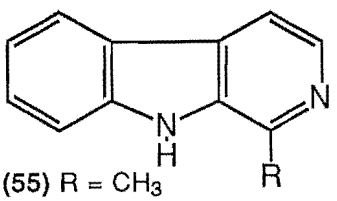


	R1	R2	R3
(108)	CHO	H	OH
(109)	CO_2CH_3	H	OH
(110)	CHO	OH	H
(111)	CO_2CH_3	H	H
(112)	CO_2CH_3	OAc	H

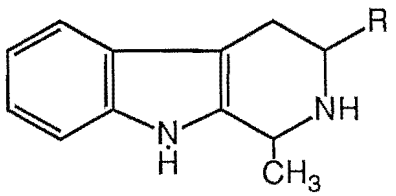
The alkaloids isolated from *Cribricellina cribraria* exhibited biological activity to widely differing degrees. In particular, as the biological activities of pavettine versus 1-ethyl-β-carboline and harman were quite different (see below), it was decided to concentrate on the synthesis of 1-substituted β-carbolines to see if any trends in their biological activities were apparent. In some cases, the identity and biological activity of the natural products could be checked against the synthetic samples. Attempts were made to relate the observed biological activities of the alkaloids to the factors considered most important in the study of structure-activity relationships.

SYNTHESES OF 1-SUBSTITUTED-β-CARBOLINE ALKALOIDS

A total of seven β-carboline alkaloids were synthesised. These were norharman (**58**), harman (**55**), 1-ethyl-β-carboline (**56**), 1-propyl-β-carboline (**113**), 1-isopropyl-β-carboline (**114**), 1-hexyl-β-carboline (**115**) and 1-phenyl-β-carboline (**116**). Most of the alkaloids were synthesised by condensation of L or DL-tryptophan and the appropriate aldehyde under acidic conditions, followed by an oxidative decarboxylation using potassium dichromate[77,88]. In the cases of 1-hexyl-β-carboline and 1-phenyl-β-carboline, the immiscibility of heptanal and benzaldehyde with water meant that the reaction had to be left to proceed for a much longer period of time with vigorous stirring. Yields in all cases were rather poor (of the order of 1-20%), and it was generally found to be more convenient to purify the products by reverse phase flash column chromatography rather than by recrystallisation.



(55) R = CH_3
(56) R = CH_2CH_3
(58) R = H
(113) R = $CH_2CH_2CH_3$
(114) R = $CH(CH_3)_2$
(115) R = $(CH_2)_5CH_3$
(116) R = C_6H_5



(117) R = CO_2H
(118) R = CO_2CH_3

Harman was prepared by an alternative method, in which tryptophan and acetaldehyde were condensed under physiological conditions of temperature and pH[89]. This allowed the isolation of the precursor of harman, 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**117**), which was subsequently converted to harman. Although this method resulted in a greatly improved yield of compound (55-60%), it was more time-consuming than the other preparation.

Methylation of the precursor with diazomethane to form methyl 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylate (**118**) was also carried out to see if this had any effect on the biological activity.

NMR SPECTROSCOPY OF β-CARBOLINE ALKALOIDS

The ^{1}H and ^{13}C nmr spectra of the β-carboline alkaloids isolated from *Cribricellina cribraria* and their derivatives, an authentic sample of pavettine (obtained from Dr. B. Charles) and of the synthetic alkaloids, were recorded for comparative purposes. The means by which the spectra of the natural product alkaloids and their derivatives were assigned are outlined in Chapter Two of this thesis. For the ^{1}H and ^{13}C nmr spectral data of these alkaloids, refer also to Chapter Two, Tables 2.1-2.2 and 2.4. The ^{1}H and ^{13}C nmr assignments for the synthetic alkaloids are summarised in Tables 3.1-3.3.

Table 3.1: ^1H nmr data for synthetic β-carboline alkaloids in ppm for $CDCl_3$ solutions.

	COMPOUND						
POSITION	**(58)**	**(55)**	**(56)**	**(113)**	**(114)**	**(115)**	**(116)**
1	8.92 s						
3	8.47 d (5.3)	8.13 d (5.2)	8.43 d (5.3)	8.39 d (5.4)	8.42 d (5.3)	8.38 d (5.2)	8.57 d (5.0)
4	7.96 d (5.3)	7.83 d (5.2)	7.83 d (5.3)	7.82 d (5.4)	7.82 d (5.3)	7.80 d (5.2)	7.92 d (5.0)
5	8.14d (7.7)	8.12 d (7.3)	8.11 d (8.0)	8.11 d (7.8)	8.12 d (8.0)	8.11 d (7.8)	8.15 d (7.9)
6	7.30 m	7.30 m	7.27 m	7.28 m	7.27 m	7.29 m	7.31 t (7.9, 7.5)
7	7.53 m	7.55 m	7.49 m	7.51 m	7.49 m	7.53m	7.50 m
8	7.53 m	7.55 m	7.49 m	7.51 m	7.49 m	7.53 m	7.50 m
9	8.6 br s	11.0 br s	9.4 br s	8.5 br s	9.1 br s		8.5 br s
1'		3.11 s	3.16 q (7.6)	3.09 t (7.8)	3.54 d septets (6.9, 1.3)	3.10 t (7.8)	
2'			1.41 t (7.6)	1.94 sx (7.8, 7.4)	1.48 d (6.9)	1.88 qt (7.8)	7.96 d (7.0)
3'				1.03 t (7.4)		1.39 m	7.58 m
4'						1.30 m	7.50 m
5'						1.30 m	7.58 m
6'						0.84 t (7.1)	7.96 d (7.0)

() coupling constants in Hz.

Table 3.2: ^{13}C nmr data for synthetic β-carboline alkaloids in ppm for $CDCl_3$ solutions.

	COMPOUND						
CARBON	**(58)**	**(55)**	**(56)**	**(113)**	**(114)**	**(115)**	**(116)**
1	133.75	141.72	146.85	145.71	150.42	145.88	143.03
3	139.38	138.86	138.84	138.80	138.52	138.62	139.61
4	114.72	112.88	112.91	112.79	112.78	112.78	113.77
4a	121.55	121.77	121.96	122.10	122.03	122.09	121.92
4b	128.96	128.32	128.63	128.59	128.76	128.66	129.84
5	121.82	121.85	121.70	121.76	121.64	121.77	121.81
6	120.20	120.20	119.92	120.06	119.92	120.09	120.27
7	128.54	128.24	128.15	128.20	128.13	128.24	128.50*
8	111.54	111.52	111.58	111.49	111.52	111.51	111.53
8a	140.39	139.99	140.34	140.06	140.17	140.10	140.31
8b	134.54	134.51	134.01	134.24	133.30	134.13	133.49
1'		20.32	27.24	36.29	31.99	34.31	138.57
2'			12.75	21.96	21.43	28.67	128.13
3'				14.19		29.45	129.22
4'						31.74	128.83*
5'						22.56	129.22
6'						14.04	128.13

* Values in vertical columns may be interchanged.

Table 3.3: ^{1}H and ^{13}C nmr data for compounds (**117**) and (**118**).

POSITION	COMPOUND (117)[a]		(118)[b]	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1	4.61 q (6.7)	49.07	4.27 dt	48.37
3	3.71 dd (4.8, 11.1)	57.70	3.84 dd (4.5, 11.2)	52.20
4	2.88 ddd (12.0, 16.1, 2.5)	23.37	2.83 ddd (11.2, 15.2, 2.5)	25.97
	3.26 ddd (4.8, 16.1, 1.3)		3.13 ddd (4.5, 15.2, 1.9)	
4a		106.83		107.49
4b		126.21		127.18
5	7.55 d (8.6)	118.08	7.47 d (7.1)	118.04
6	7.12 t	118.89	7.13 m	119.62
7	7.19 t (8.0)	121.39	7.13 m	121.78
8	7.44 d (8.0)	111.29	7.31 d (7.5)	110.84
8a		136.45		136.46
8b		132.40		135.91
9	11.20 s		7.8 br s	
1'	1.72 d (6.7)	17.08	1.51 d (6.7)	20.38
3-CO_2H		169.49		173.63
3-CO_2CH_3			3.82 s	56.60

[a] Values in ppm for DMSO solutions.
[b] Values in ppm for $CDCl_3$ solutions.
() coupling constants in Hz.

A ^{13}C nmr spectroscopy study of β-carboline alkaloids has been reported previously in the literature[65]. Nmr spectroscopic experiments on 1-ethyl-β-carboline suggested that these previously published ^{13}C nmr assignments should be revised. In the work reported here, the ^{1}H and ^{13}C nmr spectra of 1-ethyl-β-carboline (Tables 3.1 and 3.2), were assigned with the aid of COSY and HETCOR nmr experiments. Comparison of the experimental data with the literature values[65] suggested that the literature assignments of C6 and C7 for many of these alkaloids were in error.

The COSY spectrum of (**56**) obtained in $CDCl_3$, showed that the H5 doublet at 8.11 ppm was coupled to both the multiplet at 7.27 ppm and to the two proton multiplet at 7.49 ppm, so these signals were assigned as H6 and H7, H8 respectively. In turn, the multiplet at 7.49 ppm showed coupling to the H6 resonance (Table 3.4).

Proof for the assignment was given by a series of nOe experiments in DMSO-d6 (Table 3.4). Irradiation of the H5 resonance led to enhancement of the signal assigned as H6 and irradiation of this signal led to enhancement of the H5 resonance and the H7, H8 multiplet. The H6 resonance was enhanced on irradiation of the multiplet, as was the H9 resonance. These results confirmed that the assignment of the ^{1}H nmr spectrum was correct.

Table 3.4: Selected COSY and HETCOR correlations and nOe enhancements observed for 1-ethyl-β-carboline (**56**).

COSY[a]	H3	<->	H4	HETCOR[a]	H5	<->	$C_{121.70}$
	H5	<->	H6		H6	<->	$C_{119.92}$
		<->	H7		H7, 8	<->	$C_{128.15}$
	H6	<->	H7			<->	$C_{111.58}$
nOe[b]	H3	<->	H4		H7, 8	<->	H6, H9
	H4	<->	H3, H5		H9	<->	H8, H1'
	H5	<->	H4, H6		H1'	<->	H9, H2'
	H6	<->	H5, H7		H2'	<->	H1'

[a] For $CDCl_3$ solutions.
[b] For DMSO-d6 solutions.

The HETCOR nmr spectrum of (**56**) in $CDCl_3$ showed that the H6 resonance was correlated to a ^{13}C nmr signal at 119.92 ppm while the resonance arising from H7 and H8 was correlated to signals in the ^{13}C nmr spectrum at 128.15 and 111.58 ppm. This implied that the signal at 119.92 ppm arose from C6, while that at 128.15

ppm arose from C7, the reverse to the assignments given in the literature[65] for analogous β-carboline alkaloids such as harman.

As the HETCOR spectrum of (**56**) was unambiguous, the assignment of C6 and C7 had to be as given in Table 3.2. The analogous synthetic β-carboline alkaloids were therefore assigned as per these values. In cases where the ^{13}C nmr data could not be assigned by direct comparison, HETCOR and/or XCORFE nmr experiments were used to aid in the assignments.

The alkyl chain of 1-hexyl-β-carboline (**115**) was assigned in the following manner. A COSY nmr spectrum established the proton-proton connectivity throughout the chain, allowing assignment of the ^{1}H nmr spectrum. The ^{13}C nmr data was then assigned with the aid of a HETCOR nmr experiment, but the assignment of C4' and C5' was ambiguous as both were correlated to the two proton multiplet at 1.30 ppm. Unambiguous assignment of C4' and C5' was achieved by comparison of the ^{13}C nmr data with that of chimyl alcohol[90].

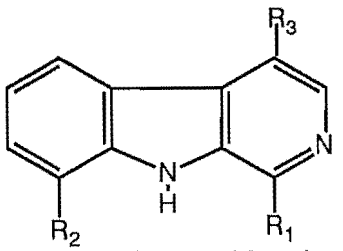
The ^{1}H nmr spectrum of 1-phenyl-β-carboline (**116**) obtained in $CDCl_3$ was difficult to assign, on account of some overlapping signals. A ^{1}H nmr spectrum of the compound was obtained at 50°C. The signals were better resolved through chemical shift difference at the higher temperature and this aided in the assignment of the spectrum at 23°C. A COSY nmr experiment confirmed the ^{1}H nmr spectral assignments. Although some of the signals in the ^{13}C nmr spectrum of (**116**) were over a very small chemical shift range, most could be unambiguously assigned with the aid of HETCOR and HMBC nmr experiments. For example, C1 and C1' were distinguished with the aid of an HMBC nmr experiment. Correlations from H3' to C1' and from H2' to C1, allowed the assignment as shown in Table 3.2.

BIOLOGICAL ACTIVITY OF β-CARBOLINE ALKALOIDS

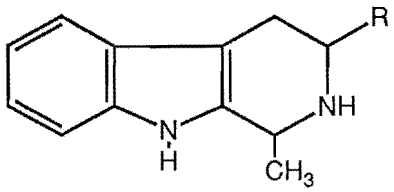
The alkaloids (**55-58**), (**82**), (**88-92**) and (**113-118**) were tested in the three biological assay systems as described in Chapter One of this thesis. For the antimicrobial/antifungal assays, compounds were submitted in a series of two-fold dilutions to determine the minimum inhibitory concentration (MIC) of the alkaloids against each organism.

1. P388/CYTOTOXICITY RESULTS

The P388 and antiviral/cytotoxicity assay results of the β-carboline alkaloids are summarised in Table 3.5. These results parallel each other, showing the same trends.



(55-58), (82), (88-92) (113-116)
For R1, R2, R3, see Table 3.5



(117) R = CO_2H
(118) R = CO_2CH_3

Table 3.5: P388 and antiviral/cytotoxicity assay results for β-carboline alkaloids.

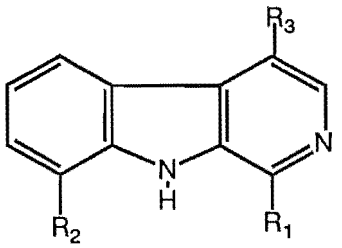
COMPOUND				P388	Antiviral/Cytotoxicity Results				
	R1	R2	R3	IC_{50}(ng/ml)	HSV1	PV1	Cyt.	Type	µg/disc
(58)	H	H	H	25 000	-	-	-		20
(55)	CH_3	H	H	25 000	-	-	-		20
(56)	CH_2CH_3	H	H	25 000	-	-	-		20
(113)	$(CH_2)_2CH_3$	H	H	25 000	-	-	-		20
(114)	$CH(CH_3)_2$	H	H	13 000	-	-	-		20
(115)	$(CH_2)_5CH_3$	H	H	9 000	±	-	±	C4	40
					-	-	±	C6	10
(116)	C_6H_5	H	H	90 000	-	-	-		20
(57)	$CH=CH_2$	H	H	100	?	?	ww	C7	2
					±	±	2+	C7	0.5
					-	-	-		0.4
(82)	$CH=CH_2$	OH	H	100	?	?	ww	C9	2
					±	±	2+	C9	0.5
					-	-	-		0.4
(88)	$CH=CH_2$	OCH_3	H	100	±	±	2+	C9	2
					±	±	±	C9	0.5
					-	-	-		0.4
(89)	CH_2CH_3	OH	H	>12 500	-	-	-		20
(90)	$CH=CH_2$	OAc	H	650	?	?	ww	C7	20
					±	±	2+	C7	5
					-	-	-		1
(91)	CH_2CH_3	OCH_3	H	>12 500	-	-	-		20
(92)	CH_2CH_3	H	SO_2CH_3	>12 500	-	-	-		20
(117)				>125 000	-	-	-		20
(118)				120 000	-	-	-		20

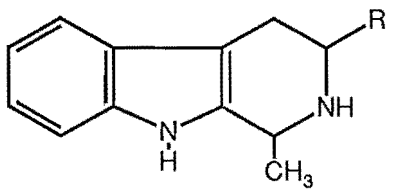
Table 3.5 clearly shows that for the P388 and antiviral/cytotoxicity assays, only those alkaloids with a vinyl sidechain at the 1-position exhibited any significant cytotoxicity or P388 activity. For these alkaloids, the presence or absence of a substituent at the 8-position had no effect on the cytotoxicity of the alkaloid, with pavettine (**57**) and 1-vinyl-8-hydroxy-β-carboline (**82**) having essentially the same activity. An exception to this is 1-vinyl-8-acetoxy-β-carboline (**90**) which was less active than the other alkaloids by a factor of six. All of the alkaloids with an unsaturated alkyl sidechain at position 1 exhibited no significant P388 activity or cytotoxicity, having IC_{50} values greater than 12 500 ng/ml. An exception to this is 1-hexyl-β-carboline, although the IC_{50} value of this compound of 9 000 ng/ml is much higher than those of the vinyl alkaloids and the cytotoxicity is barely discernible at the concentrations tested.

2. ANTIMICROBIAL/ANTIFUNGAL ASSAY RESULTS

The results of the antimicrobial/antifungal assay are summarised in Table 3.6. The antimicrobial/antifungal assay results are significantly different from those of the other assay systems.

At the concentrations tested, none of the alkaloids tested showed any significant activity (<60 μg/disc) against *Pseudomonas aeruginosa* and 1-phenyl-β-carboline (**116**) and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**117**) showed no activity against any of the microorganisms. Norharman (**58**) showed quite strong activity against *E. coli*. The only other alkaloids to show activity against this organism were pavettine (**57**), 1-ethyl-8-hydroxy-β-carboline (**89**) and harman (**55**). This activity was rather weak for the former two compounds and very weak for harman. The most active alkaloid against *Bacillus subtilis* was pavettine (**57**) with a MIC of 1.9-3.8 μg/disc. 1-Ethyl-8-methoxy-β-carboline (**91**), 1-propyl-β-carboline (**113**), 1-phenyl-β-carboline (**116**), 1-hexyl-β-carboline (**115**) and the tetrahydro-β-carbolines were all inactive against this organism. Most of the remaining alkaloids, namely norharman (**58**), harman (**55**), 1-ethyl-β-carboline (**56**), 1-isopropyl-β-carboline (**114**), 1-vinyl-8-hydroxy-β-carboline, (**82**) 1-vinyl-8-methoxy-β-carboline (**88**) and 1-vinyl-8-acetoxy-β-carboline (**90**) exhibited a MIC of 7.5-15 μg/disc.





(117) R = CO_2H
(118) R = CO_2CH_3

	R1	R2	R3		R1	R2	R3
(57)	$CH=CH_2$	H	H	**(58)**	H	H	H
(82)	$CH=CH_2$	OH	H	**(55)**	CH_3	H	H
(88)	$CH=CH_2$	OCH_3	H	**(56)**	CH_2CH_3	H	H
(89)	CH_2CH_3	OH	H	**(113)**	$(CH_2)_2CH_3$	H	H
(90)	$CH=CH_2$	OAc	H	**(114)**	$CH(CH_3)_2$	H	H
(91)	CH_2CH_3	OCH_3	H	**(115)**	$(CH_2)_5CH_3$	H	H
(92)	CH_2CH_3	H	SO_2CH_3	**(116)**	C_6H_5	H	H

Table 3.6: Antimicrobial/antifungal activities of β-carboline alkaloids, expressed as minimum inhibitory concentration (MIC) in µg/disc.

COMPOUND	Ec	Bs	Pa	Ca	Tm	Cr
(58)	3.7-7.5	7.5-15	>60	1.9-3.8	0.9-1.9	15-30
(55)	60-120	7.5-15	>60	1.9-3.8	3.7-7.5	15-30
(56)	>120	7.5-15	>60	1.9-3.8	1.9-3.8	30-60
(113)	>120	>120	>60	>120	15-30	>120
(114)	>120	7.5-15	>60	1.9-3.8	0.45-0.9	15-30
(115)	>60	>60	>60	>60	1.9-3.8	7.5-15
(116)	>60	>60	>60	>60	>60	>60
(57)	30-60	1.9-3.8	>60	1.9-3.8	0.1-0.2	0.9-1.9
(82)	>60	7.5-15	>60	15-30	0.45-0.9	3.7-7.5
(88)	>60	7.5-15	>60	7.5-15	0.45-0.9	15-30
(89)	30-60	30-60	>60	30-60	30-60	>60
(90)	>60	7.5-15	>60	15-30	0.9-1.9	>60
(91)	>60	>60	>60	7.5-15	7.5-15	>60
(92)	>60	30-60	>60	>60	30-60	>60
(117)	>60	>60	>60	>60	>60	>60
(118)	>60	>60	>60	7.5-15	>60	>60

Ec = *E. coli*, Bs = *B. subtilis*, Pa = *P. aeruginosa*, Ca = *C. albicans*, Tm = *T. mentagrophytes* and Cr = *C. resinae*.

Against the fungus *Candida albicans*, the lowest observed MIC was 1.9-3.8 µg/disc and this was exhibited by norharman (**58**), harman (**55**), 1-ethyl-β-carboline (**56**), 1-isopropyl-β-carboline (**114**) and pavettine (**57**). All the vinyl alkaloids with aromatic substitution were less active. Again, 1-ethyl-8-hydroxy-β-carboline (**89**) exhibited only weak activity (30-60 µg/disc) and all other compounds were inactive, with the exception of methyl 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylate (**118**), which had a MIC of 7.5-15 µg/disc. *Candida albicans* was the only organism which this compound exhibited any significant activity against.

Pavettine (**57**) was the most active alkaloid against the dermatophyte, *Trichophyton mentagrophytes*, with a MIC of 0.1-0.2 µg/disc. This was the lowest MIC found for any of the alkaloids against any of the microorganisms tested. Again, the other vinyl alkaloids were less active, but these were still quite potent. Of the saturated 1-alkyl-β-carboline alkaloids, 1-isopropyl-β-carboline (**114**) was the most active, followed by norharman (**58**), 1-ethyl-β-carboline (**56**) and 1-hexyl-β-carboline (**115**), harman (**55**) and 1-propyl-β-carboline (**113**). This was the only microorganism against which 1-propyl-β-carboline exhibited any measurable activity. 1-Ethyl-8-methoxy-β-carboline (**91**) had a MIC of 7.5-15 µg/disc, while both 1-ethyl-8-hydroxy-β-carboline (**89**) and 1-ethyl-4-methylsulphone-β-carboline (**92**) had weak activity at 30-60 µg/disc. The other alkaloids were inactive at the concentrations tested.

Many of the alkaloids showed either greatly reduced activity or no activity against *Cladisporum resinae*. Pavettine (**57**) was still the most effective compound and 1-vinyl-8-hydroxy-β-carboline (**82**) was less active by a factor of four. The other 8-substituted alkaloids however, were much less active again. 1-Hexyl-β-carboline (**115**) had a MIC of 7.5-15 µg/disc but the growth of the fungus was only retarded to a very slight degree by this compound, rather than significantly affected.

3. STRUCTURE/ACTIVITY RELATIONSHIPS

Nowadays, the three factors which are considered most relevant in the study of structure-activity relationships are solubility, ionisation or electron distribution and shape or steric fit[91]. These three factors are now examined independently.

a) Shape.

A ^1H nmr spectroscopy study of the binding mode and kinetics of antitumour drugs to DNA has shown that β-carboline alkaloids bind to DNA by intercalation[92]. Intercalating compounds bind to DNA by sliding between the base pairs as shown in Figure 3.1.

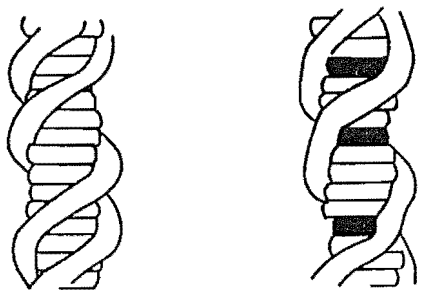
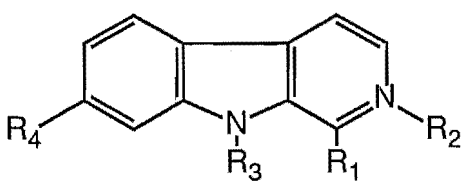


Figure 3.1: DNA, showing the secondary structure and intercalation of molecules between the base pairs.

This binding causes the unwinding of the DNA helix. An intercalating molecule must possess a large, flat area of approximately 38 $Å^2$ to be able to fit between the base pairs of DNA[93]. A further requirement is a positive charge, as the space available for intercalation is negatively charged, due to the surrounding phosphate anions, purine and pyrimidine bases and oxygen atoms of deoxyribose[93]. The β-carboline alkaloids are essentially planar, thus fulfilling the requirement of a large flat area. The requirement of positive charge is fulfilled by the indolic proton. The ^1H nmr spectroscopy study states that the "possession of three or more fused benzenoid rings virtually assures that the compound will bind primarily by intercalation of that chromophore, in the absence of bulky substituents that prevent intercalative binding"[92]. The β-carboline alkaloids used in the study were norharman (**58**), methylnorharman (**119**), methylharman (**120**) and methylharmine (**121**). For these compounds, intercalative binding was shown to be guanidine-cytosine base-pair specific and the compounds were also shown to be in fast exchange with the DNA, that is, the residence time of the compounds at any specific DNA binding site is short.



	R1	R2	R3	R4
(119)	H	CH_3	CH_3	H
(120)	CH_3	CH_3	CH_3	H
(121)	CH_3	CH_3	CH_3	OCH_3

The study concluded that "... at least for the intercalating compounds, cytotoxicity and useful antitumour activity are associated with long drug/DNA residence times"[92]. It is thought that the cytotoxicity of DNA drugs is a result of the induction of strand breaks in the DNA and of the blocking of polymerase enzymes during replication and transcription, therefore compounds with longer residence times at a DNA binding site may have higher cytotoxicity and antitumour activity[92]. The results of the study bear this out, showing that structural changes resulting in a slowing of the exchange kinetics generate antitumour activity.

The structural features determining the kinetics of binding of a compound to DNA are not as well defined as those that determine if a compound binds by intercalation. As pointed out in the study, the attachment of cationic sidechains to an intercalating compound might increase residence time by providing additional electrostatic interactions between the compound and the DNA binding site, but the authors conclude that the effect also depends on the position, length and structure of the sidechain[92].

The results of the current study appear to be in line with these findings.

b) Electronic factors

The lack of P388 activity for the 1-alkyl-β-carbolines cannot be on account of a decrease in flat area of the molecule, as in for example, replacing the vinyl substituent with an ethyl substituent, as the β-carboline nucleus itself has enough flat area to bind by intercalation to DNA as was proven for norharman in the ^{1}H nmr study. Nor is it necessarily a result of the presence of a bulky alkyl group preventing intercalation by steric constraint, as methylharman was also shown to bind by intercalation in the ^{1}H nmr study, yet it is not cytotoxic. It is more likely that the alkaloids lacking a vinyl sidechain still bind to DNA by intercalation but are in fast exchange, as was shown for compounds (**58**) and (**119-121**) in the study, and thus have no cytotoxic or antitumour properties. Changing the substituent at position 1 from vinyl to alkyl had two effects, namely loss of conjugation and loss of planarity. As already stated, the loss of planarity would not be expected to affect intercalative binding in this case, so it would seem that the extension of conjugation in these compounds may be an important factor in determining activity. On this basis, 1-phenyl-β-carboline might have been expected to exhibit similar cytotoxic and P388 activity to the vinyl alkaloids, as there may be an extension of conjugation through the

phenyl ring. However, there is a steric constraint to the phenyl group being co-planar with the rest of the molecule[94] which precludes this and accordingly 1-phenyl-β-carboline is inactive in these assay systems.

Another possible explanation as to why the 1-vinyl group is essential for antitumour/cytotoxic activity in the β-carboline alkaloids, is on account of the reactivity it imparts to the alkaloids containing it. The 1-vinyl alkaloids are more susceptible to undergoing reaction with an electrophile as shown in Figure 3.2.

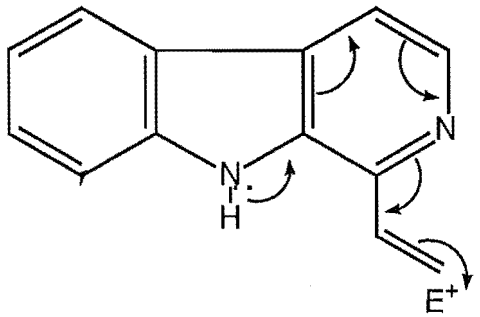
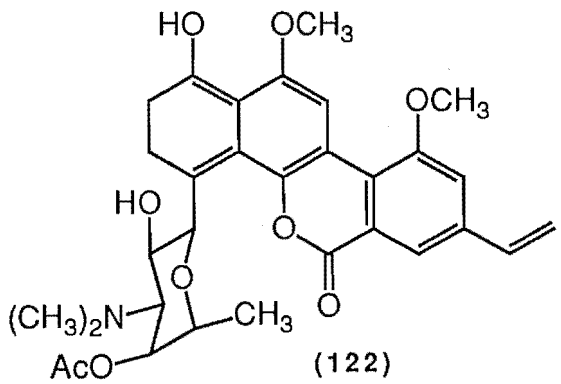
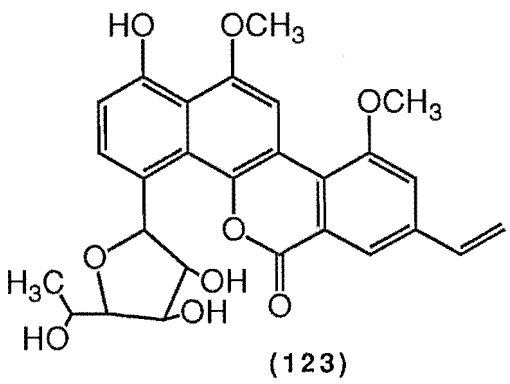


Figure 3.2: Possible reaction of 1-vinyl-β-carboline alkaloids with an electrophile.

This could conceivably occur during intercalation and may result in compounds with higher affinities for DNA and thus better antitumour/cytotoxic activity. It is noteworthy that for the antibiotic ravidomycin (**122**) a similar trend was observed, in that the vinyl group appeared to be necessary for antitumour activity. Replacing the vinyl group with an ethyl group significantly lowered the activity[95]. This was also the case for the related antibiotic, gilvocarcin V (**123**)[96]. Thus, for the antitumour and antiviral/cytotoxicity properties of these alkaloids, electronic effects would seem to be the most important factor.



(122)



(123)

Trends in the antimicrobial and antifungal activities of the alkaloids are less evident, but they clearly do not fit this pattern. The observed trends vary depending

upon the organism tested against. However pavettine was generally the most active compound, except against *E. coli* where norharman was by far the most potent. Aromatic substitution resulted in decreased activity in almost all cases and replacing a vinyl group with an ethyl group generally decreased the activity. This finding is in agreement with that of a study of quasi-dimeric indole alkaloids which found that aromatic substitution decreased the antimicrobial/antifungal activity[97]. 1-Ethyl-8-hydroxy-β-carboline (**89**), 1-ethyl-8-methoxy-β-carboline (**91**) and 1-ethyl-4-methylsulphone-β-carboline (**92**) are therefore doubly deactivated with respect to pavettine and accordingly would be expected to have greatly reduced activity, as is indeed the case.

For the 1-substituted alkaloids, activity dropped off dramatically between 1-ethyl-β-carboline (**56**) and 1-propyl-β-carboline (**113**), although 1-isopropyl-β-carboline (**114**) was at least as active as 1-ethyl-β-carboline. 1-Hexyl-β-carboline (**115**) might therefore have been expected to be very inactive and this was generally the case, but it exhibited reasonably strong activity against *T. mentagrophytes* and weak activity against *C. resinae*.

As the β-carboline alkaloids are known DNA intercalators, it is not surprising that they generally exhibit good antimicrobial and antifungal activity. DNA in bacteria is present as a single chromosome which is often circular and laced through the plasma membrane[98]. The solitary chromosome is much more vulnerable than in higher organisms where DNA is membrane protected inside the cell nucleus[98]. Also, intercalating compounds are known to prefer circular DNA[93] and in general, although bacterial DNA only constitutes 2-3% of the cell dry weight, it comprises approximately 10% of the volume[99], making it relatively accessible to potential reactant molecules.

c) Solubility

As shape and electronic effects would not seem to be the only determining factors in the observed antimicrobial/antifungal activities of the alkaloids; solubility, the third important factor in the study of structure-activity relationships may be of more significance here. Even if the solubility of a class of drugs is not the main factor responsible for their action, it still controls membrane penetration into cells. A favourable balance between lipophilic and hydrophilic properties may ensure that a drug reaches its site of action[100]. Bacteria generally have membranes which permit simple diffusion across them[100]. These membranes hinder the passage of ions and permit that of neutral molecules. Compounds with high oil/water partition coefficients

and thus high lipophilicity, diffuse the fastest. However, if the partition coefficient is too high, a compound may enter the membrane freely but be trapped there as its affinity is too great[100].

The standard method for measuring the partition coefficient of a compound uses octanol and water. The substance is equilibrated between these two phases by rapid shaking, centrifuged to clear emulsions and the concentration of substance in each phase determined[101]. The partition coefficient, P of a substance is defined as:

$$P = [\text{octanol}] / [\text{water}]$$

where [octanol] is the concentration of substance in the octanol layer and [water] is the concentration of substance in the water layer. Log P is the value usually quoted in the literature.

Attempts were made to measure the partition coefficients of some of the β-carboline alkaloids by this shaking method, to see if any correlation between the partition coefficients and the observed antimicrobial/antifungal activities existed. The amount of compound in each phase was determined by uv spectroscopy. The limited supply of compounds available for use, combined with experimental difficulties meant that the measurements could not be accurately performed on most of the alkaloids. The major experimental difficulty was the low solubility of some of the alkaloids in one or both phases. For example, 1-phenyl-β-carboline was barely soluble in either phase and any compound that did dissolve formed an emulsion at the interface, which centrifugation and prolonged standing failed to clear. Also, when log P is greater than 4, the reliability of this method is a problem[100]. It is possible to use a procedure defined by Hansch *et al.*[101] to calculate partition coefficients of compounds in a series from that of a parent compound, using either the π values for a substituent[101] or the fragmental constant, *f*[102]. The substituent constant, π, is derived from partition coefficient measurements of many compounds between octanol and water and is defined as : $\pi = \log P_x - \log P_H$, where P_x is the partition coefficient of a derivative and P_H is that of the parent compound. π Values do not take account of thermodynamic properties however, and thus π_{methyl} and $\pi_{methylene}$ for example, are assumed to be the same[100]. The hydrophobic fragmental constant, *f*, is similar to π, but attempts to overcome this error. The *f* values were calculated from published values of log P in octanol/water. For example $f_{CH3} = \log P_{\text{toluene}} - f_{phenyl}$[100]. The partition coefficient of 1-propyl-β-carboline was able to be accurately measured by the rapid shaking method and log P was determined to be 2.50. This value was used

to calculate the partition coefficients for the homologous series of straight chain saturated 1-alkyl-β-carboline alkaloids, using both π values and f values for the substituents (see Work Described in Chapter Three, p. 165). The results of these calculations are summarised in Table 3.7.

Table 3.7: Calculated log P values for straight chain saturated 1-alkyl-β-carboline alkaloids.

COMPOUND	log P calculated from: π values[101]	f values[102]	log P experimental
(58)	1.00	0.52	2.26 ± 0.41
(55)	1.50	1.18	2.33 ± 0.42
(56)	2.00	1.84	2.57 ± 0.46
(113)	2.50*± 0.23	2.50*± 0.23	2.50 ± 0.23
(115)	4.00	4.48	not determined

* value determined experimentally and used as the basis for calculations.

From the results in Table 3.7, it is clear that the calculated values for log P differ somewhat, depending on the particular constants used, but the log P value increases with increasing chain length (Figure 3.3).

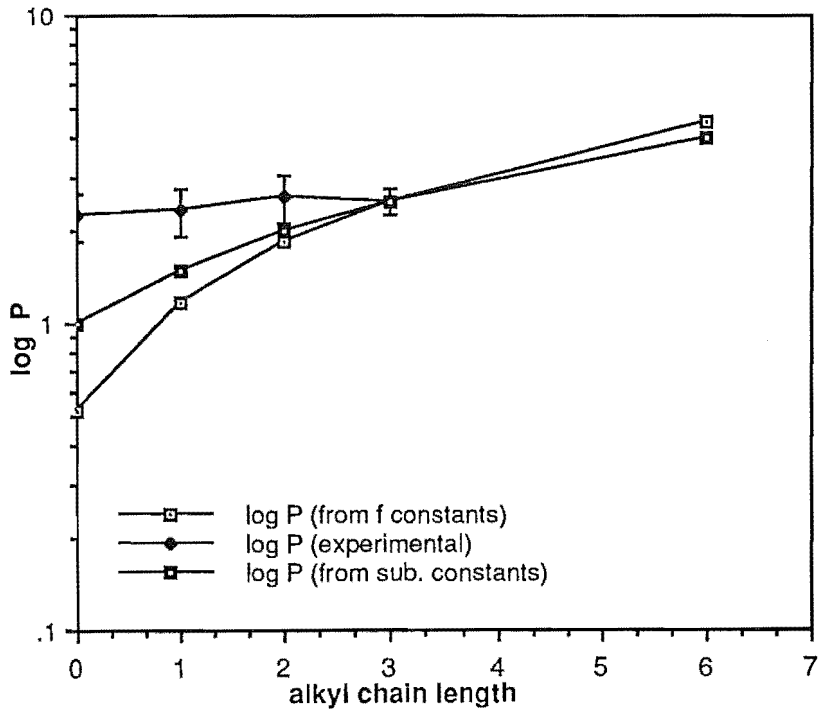


Figure 3.3: A plot of chain length in straight chain saturated 1-alkyl-β-carboline alkaloids versus log P values (calculated and experimental).

However, this procedure cannot completely substitute for experimental measurements, as it assumes no group interactions take place[102,103]. This is evidenced by the fact that although no accurate values of log P were determined for the alkaloids, one-off experimental trials indicated that these log P values would fall between 2.0 and 3.0, a result inconsistent with that of calculation by either method.

Alternative methods of assessing the relative solubilities were also investigated. The Rf values for the alkaloids on normal phase tlc in two different solvent systems and the reverse phase hplc retention indices of the compounds were measured to see if any trends between these parameters and the observed antimicrobial/antifungal activities were evident. Both of these methods have been used by other workers to determine partition coefficients of compounds, where the rapid shaking method of measurement is impossible or impractical. Reverse phase tlc[103-106] and hplc[107-109] methods have been used. In the current study, normal phase tlc and reverse phase hplc were employed. It was concluded from the results of a previous study of the tlc properties of β-carboline alkaloids[110], that chloroform/methanol (9:1) was a very good solvent system for these compounds. In the current work, ethyl acetate/acetone (1:1) had proven to be a useful solvent system for tlc analysis of these alkaloids. Rf values for normal phase tlc on silica gel were therefore determined for these two solvent systems. Reverse phase hplc retention indices for the alkaloids were determined using a PRP-1 column (analogous to C18) in methanol/water-TFA (3:1). Assuming that no absorption occurs, the Rf values and the hplc retention indices should be linearly related to the partition coefficient. The Rf values for the alkaloids and the hplc retention indices are summarised in Table 3.8. These values were determined from multiple measurements for each system.

As can be seen from the data, the order of elution for some of the alkaloids varies depending on the solvent system and type of chromatography employed. 1-Ethyl-4-methylsulphone-β-carboline (**92**) for example, has a retention time on hplc inconsistent with that expected from its Rf values on tlc and with its observed place in the elution order of the natural product alkaloids on reverse phase flash column chromatography. This could indicate that the compound is being absorbed to the hplc column, or that it has unusual solubility properties, which might not be unexpected from its structure. Other compounds with somewhat varying elution orders between the systems are 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**117**), methyl 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylate (**118**) and

1-hexyl-β-carboline (**115**). It is interesting to note that all of these compounds are relatively inactive.

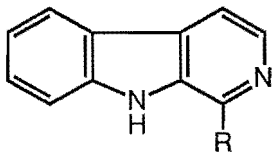
Table 3.8: Rf values and hplc retention indices for β-carboline alkaloids.

COMPOUND	Rf[a]	Rf[b]	T_c-T_o/T_o[c]
(58)	0.18	0.3	0.29
(117)	0.20	0.27	0.61
(55)	0.20	0.29	0.32
(89)	0.31	0.23	0.43
(56)	0.36	0.36	0.50
(91)	0.37	0.34	0.65
(118)	0.42	0.47	0.87
(113)	0.43	0.40	0.71
(82)	0.51	0.35	0.60
(90)	0.51	0.37	0.62
(57)	0.53	0.43	1.28
(115)	0.53	0.47	0.78
(92)	0.54	0.42	2.03
(114)	0.54	0.44	0.80
(88)	0.55	0.52	1.64
(116)	0.58	0.56	1.94

[a]Ethyl acetate/acetone (1:1) on silica gel.
[b]Chloroform/methanol (9:1) on silica gel.
[c]Methanol/water-TFA (3:1). T_c = retention time (seconds) for each alkaloid, T_o = retention time for a non-retained compound (methanol).

The data for each of the systems was examined to see if any correlation existed between the partition coefficients for the alkaloids in these systems and the observed antimicrobial/antifungal activities. No general trend was observed between Rf or hplc retention index and activity for all the alkaloids taken as a series. This is perhaps not surprising, as if the alkaloids studied are simply ordered according to their partition coefficients, this represents the alteration of more than one feature of the structure at a time.

If the alkaloids were considered to be several smaller series however, some trends between partition coefficient and observed antimicrobial/antifungal activities were apparent. As previously stated, these trends were not always consistent for each organism tested against, but even so, some general trends could be inferred. For the series of straight chain saturated 1-alkyl-β-carboline alkaloids, an increase in Rf value or hplc retention index in the systems used (and thus an increase in partition coefficient) resulted in a decrease in antimicrobial/antifungal activity. A graph of the hplc retention indices of these alkaloids versus their minimum inhibitory concentrations against *C. resinae* in μg per disc (Figure 3.4) serves to illustate this trend.



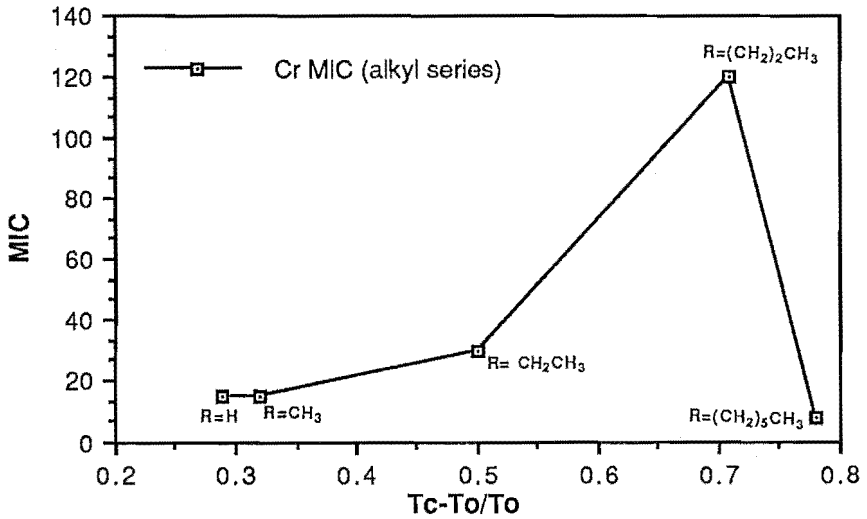
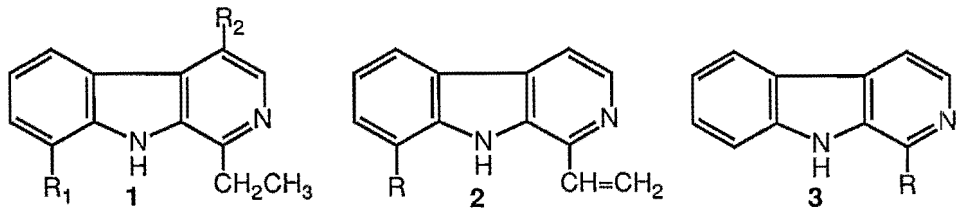


Figure 3.4: Hplc retention indices versus MIC (μg/disc) against *C. resinae* for straight chain saturated 1-alkyl-β-carboline alkaloids.

For this series the activity did not increase until an optimum value was reached and then decline, but rather the lower members of the series generally exhibited the same or similar activities until a threshold was reached at 1-propyl-β-carboline, and then the activity dropped off dramatically. The activity of 1-hexyl-β-carboline against *T. mentagrophytes and C. resinae* was an exception to this. The activity of 1-isopropyl-β-carboline was of a similar level to that observed for the lower members of the straight chain series. Tlc and hplc measurements indicated that this alkaloid should have a higher partition coefficient than 1-propyl-β-carboline, but experimental

measurements and calculation indicated that the partition coefficient is actually lower than that of 1-propyl-β-carboline, which is an interesting anomaly.

If the 1-ethyl and 1-vinyl substituted alkaloids are considered as two separate series, it can be seen that for each, the optimum activity was reached at the unsubstituted compound. Activity declined with aromatic substitution, which resulted in an increase or decrease in partition coefficient. Examples of this parabolic relationship between solubility and antimicrobial activity have been previously reported[104]. A typical example of this trend as it applies to the alkaloids here is shown in Figure 3.5.



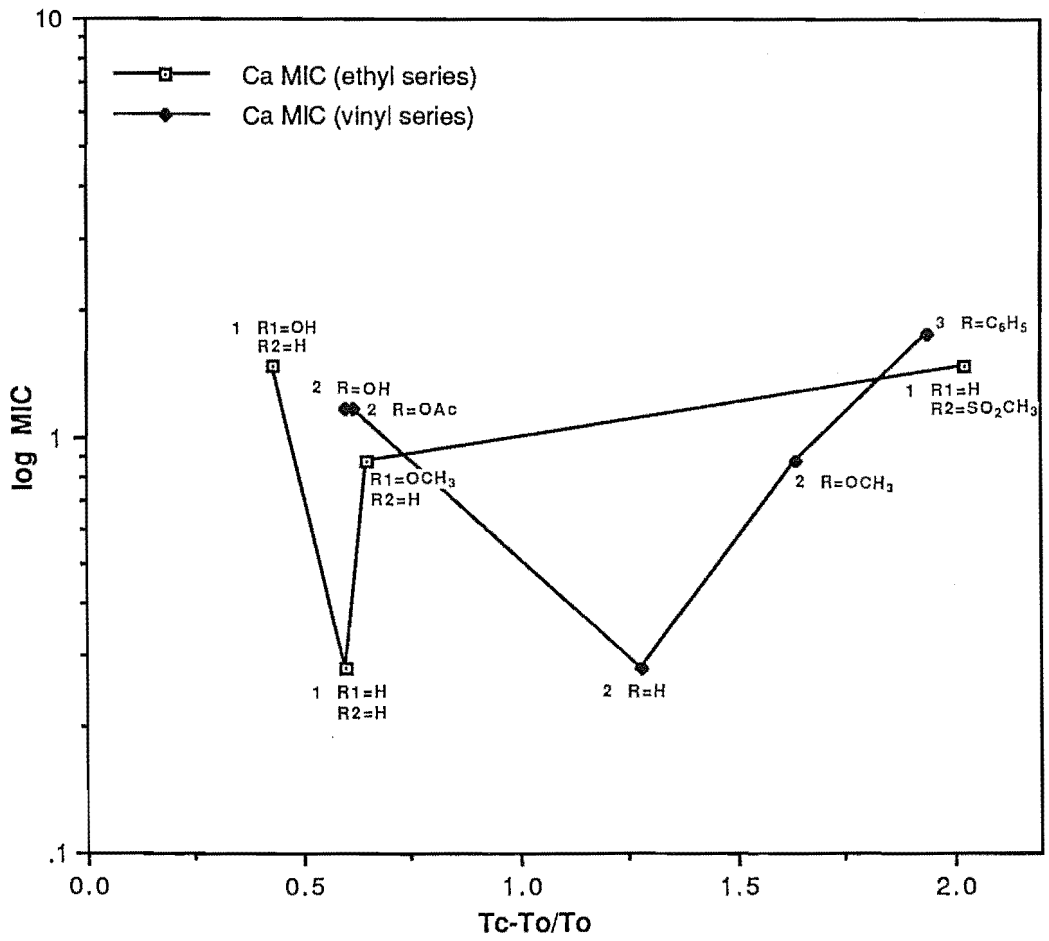


Figure 3.5: Hplc retention index versus log MIC against *C. albicans* for the 1-ethyl and 1-vinyl β-carboline alkaloid series.

Obviously aromatic substitution could affect other factors besides solubility, but in general, vinyl substituted alkaloids were at least as active as 1-ethyl-β-carboline and had higher partition coefficients, so solubility obviously plays some role in the antimicrobial/antifungal activities of these alkaloids.

There is generally good correlation between the data obtained by the tlc and hplc methods with that obtained by direct measurement of partition coefficients, indicating that these are indeed useful alternative methods of obtaining structure-activity data.

CONCLUDING REMARKS

Some general conclusions can be drawn regarding the biological activity of the β-carboline alkaloids, in light of the results obtained in the current study and those of other research workers.

The mode of action of the β-carboline alkaloids is DNA intercalation. The β-carboline nucleus contains the flat area of 38 $Å^2$, required for a compound to bind to DNA by intercalation and that intercalation is indeed the mode of binding of these alkaloids to DNA has been shown by a ^1H nmr spectroscopy study[92].

A vinyl group is necessary for good antitumour/cytotoxic activity of these alkaloids, either because of electronic effects or an increase in reactivity. The importance of the 1-vinyl group for antitumour/cytotoxic activity is most likely on account of the reactivity it imparts to the compounds containing it. If the activity was simply due to extended conjugation outside the β-carboline nucleus, 1-phenyl-β-carboline would be expected to exhibit good antitumour/cytotoxic activity and this is not the case. A terminal vinyl group is also essential for the antitumour/cytotoxic properties of the antibiotics ravidomycin[95] and gilvocarcin V[96].

In the current study, the antimicrobial/antifungal activity of the straight chain saturated 1-alkyl-β-carboline alkaloids generally decreases with increasing chain length. Activities of the lower members of the series were of a similar level, but the activity declined sharply at 1-propyl-β-carboline. Results of a Japanese study of the antifungal activities of 1-alkyl-β-carbolines against *Trichophyton interdigitale* are in contrast to the current findings. Alkaloids with the 1-alkyl substituent varying from a hydrogen to a decyl chain were tested, along with some branched chain substituted alkaloids[87]. The study found that the branched chain alkaloids had the same MIC as the equivalent straight chain compounds, although this is clearly not the case here for 1-propyl-β-carboline and 1-isopropyl-β-carboline. The study also found that harman,

1-ethyl-β-carboline and 1-propyl-β-carboline all had the same MIC and that this was twice as high as that of norharman. From that point down the series however, the longer the alkyl chain, the more active the alkaloid. For the *Trichophyton* species used at Canterbury, 1-propyl-β-carboline was considerably less active than 1-ethyl-β-carboline and 1-hexyl-β-carboline had the same MIC as 1-ethyl-β-carboline.

Aromatic subtitution of the β-carboline alkaloids results in a decrease in antimicrobial/antifungal activity, as does replacing a vinyl with an ethyl group. This finding is in agreement with that of a study of quasi-dimeric alkaloids[97]. This previous study also tested the activity of harman against some of the same organisms used in the Canterbury Marine Chemistry group's assay system, namely *B. subtilis*, *E. coli*, *P. aeruginosa* and *C. albicans* and found it to be inactive against all of these organisms at the concentrations tested. In the current study however, harman exhibited good antimicrobial/antifungal activity and its antifungal properties have been reported elsewhere[87,111], so clearly there is something inconsistent with the results of the study which found it to be inactive.

Solubility is at least part of the reason for the observed antimicrobial/antifungal activities of these alkaloids. The results of the current study indicate that the antimicrobial/antifungal activity of the alkaloids is not entirely due to their solubility properties, but the observed results show some relationship between solubility and activity within the 1-alkyl-β-carboline, the 1-ethyl-β-carboline and the 1-vinyl-β-carboline series.

CHAPTER FOUR

4.1 BROMOPYRROLE COMPOUNDS FROM SPONGES -A REVIEW

Over the past few years, a number of unusual bromopyrrole derivatives have been isolated from marine sponges. Many of the compounds were isolated almost simultaneously by different groups of workers and have been given different trivial names, rendering the literature somewhat confusing. Another point of confusion in the literature has been the taxonomy of the bromopyrrole-containing sponges. Figure 4.1 shows the taxonomic relationships between sponges from which these compounds have been isolated.

Class

Demospongiae

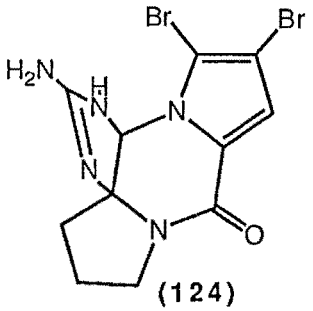
Subclass	**Order**	**Family**	**Genus**
Tetractinomorpha	Axinellida	Axinellidae	*Axinella* *Phakellia* *Acanthella* *Pseudaxinyssa*
		Agelasidae	*Agelas*
Ceractinomorpha	Halichondrida	Hymeniacidonidae	*Hymeniacidon*
	Poecilosclerida	Myxillidae	*Lissodendoryx*

Figure 4.1: Taxonomic relationships between bromopyrrole-containing sponges.

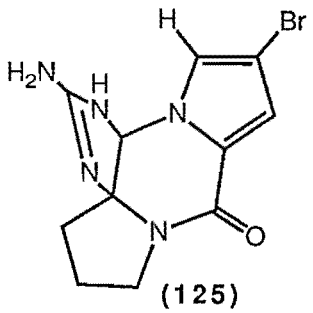
Most of the compounds have been isolated from sponges of the order Axinellida and the majority of them from the family Agelasidae which contains only the genus *Agelas*. In their 1983 review on the chemotaxonomy of the Porifera, Bergquist and Wells state that all the species of *Agelas* examined to date have yielded bromopyrroles but that they are not widespread among the family Axinellidae[112]. They also comment on the similar amino acid patterns in *Agelas* species and some Axinellidae. Some bromopyrrole compounds have been found in *Hymeniacidon* species and Bergquist and Wells note that some morphological characteristics of the Axinellidae parallel those of the Hymeniacidonidae and that there are some

similarities in sterol patterns such as the more unusual A-norstanols[112]. This raises the question of whether the isolation of bromopyrroles from two different orders is a case of mistaken identity or implies a closer relationship between the families Axinellidae and Hymeniacidonidae than their classification into the orders Axinellida and Halichondrida respectively imply. Considering the confusion which exists over these compounds, a chronological review of the isolation and chemistry of the bromopyrroles and related compounds is in order. Chevolot published a small review on oroidin and related compounds in 1981[113]. This review encompasses the compounds discussed in Chevolot's review and includes other bromopyrrole compounds isolated since.

The first report of the isolation of a bromopyrrole was from a Great Barrier Reef sponge, *Phakellia flabellata*, (Axinellidae), when Sharma and Burkholder isolated dibromophakellin (**124**), and monobromophakellin (**125**), both with broad spectrum antimicrobial activity[114,115]. Compound (**124**) was isolated as the enantiomer (-)-dibromophakellin and an X-ray crystal structure analysis of the monoacetate was obtained.

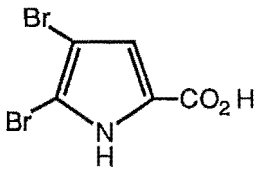


(124)



(125)

In 1971 Minale *et al.* reported the presence of 4,5-dibromo-pyrrole-2-carboxylic acid (**126**), and its nitrile (**127**) and amide (**128**) in the sponge *Agelas oroides*[116]. The corresponding methyl ester (**129**) was also found but this was an artefact arising from the use of methanol in the isolation procedure. Refluxing of the nitrile, (**127**) under basic conditions, (20% KOH), yielded both (**126**) and (**128**) but only (**126**) after a longer period of time. They also found oroidin (**130**) and prepared the N-acetyl derivative and the dihydro equivalent of this, both of which on base hydrolysis yielded (**126**) and (**128**). Unfortunately their structural assignment was incorrect. The structure was later clarified by Garcia *et al.* who synthesised the dihydroacetate[117]. Minale *et al.* subsequently found oroidin in two other species of *Axinella* : *Axinella verrucosa* and *Axinella damicornis* [118].



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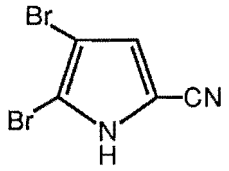

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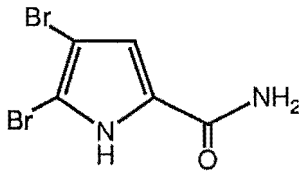

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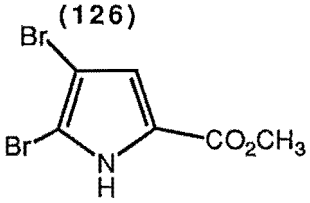

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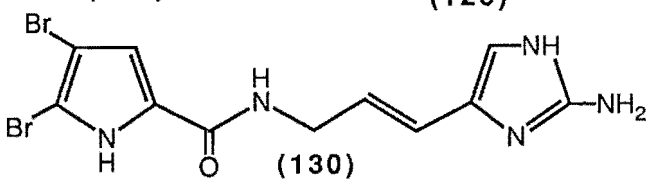
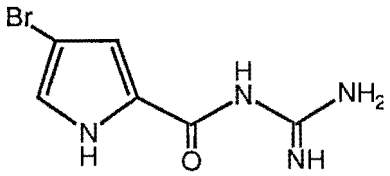


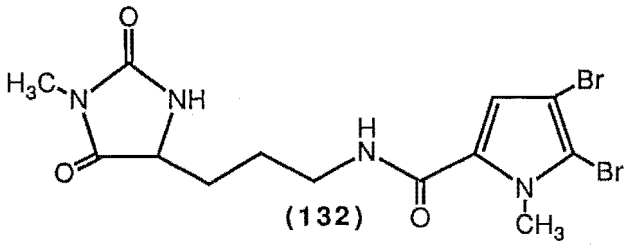
(130)

Chib *et al.* isolated the guanidine compound (**131**) from an unidentified species of *Agelas* and found it to have antimicrobial and antibiotic properties[119]. Later they synthesised some thiophene and furan guanidine compounds, some of which showed moderate antimicrobial activity, but not to the same extent as that of a series of synthetic pyrrole derivatives[120].

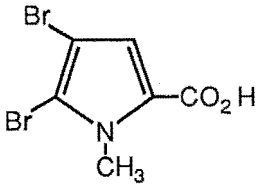


(131)

The compound midpacamide (**132**), isolated by Scheuer *et al.* from an unidentified sponge from the Marshall Islands, differs slightly from others of its class in that it contains a 3-methylhydantoyl moiety. 1-Methyl-4,5-dibromopyrrole-2-carboxylic acid (**133**), was also isolated from the sponge and synthesised as proof of structure. Base hydrolysis of midpacamide yielded (**133**)[121].



(132)



(133)

In 1977 Sharma and Magdoff-Fairchild gave a much fuller account of the isolation and structural determination of (**124**) and (**125**) and suggested that the reason for their low basicity in comparison to other guanidines is on account of inhibition of resonance in the resulting cations, due to the twisted conformation of the imidazoline ring[122]. For guanidines, strong basicity is a result of resonance stabilisation of the guanidinium ion formed (Figure 4.2).

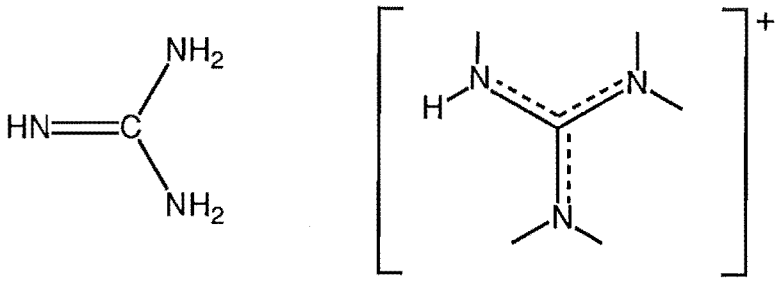
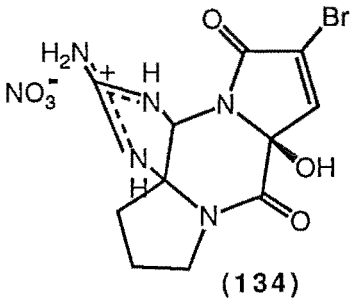
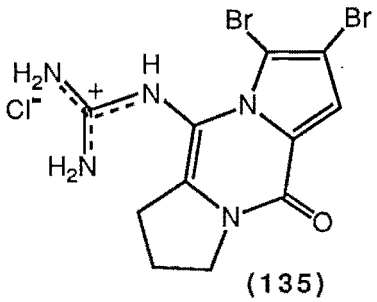


Figure 4.2: Guanidine and the guanidinium cation, showing the resonance stabilisation of the cation.

To achieve this, the three guanidine nitrogen atoms must be coplanar. In compounds (**124**) and (**125**) the nitrogens are not coplanar, as the imidazoline ring is twisted and if coplanarity were to be achieved, strong ring strain of the central six-membered ring would occur[122]. Oxidation of (**124**) with dilute nitric acid and treatment with dilute hydrochloric acid gave respectively compounds (**134**) and (**135**) which were prepared to substantiate this hypothesis[122].

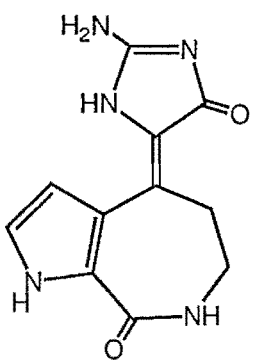


(**134**)

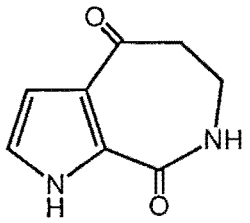


(**135**)

Sharma *et al.* then outlined the partial structure of another compound from *Phakellia flabellata*[123] and later published the full structure[124]. Like the phakellins (**124**) and (**125**), the compound occurs naturally as a hydrochloride, which on treatment with sodium carbonate yields the free base form, (**136**). The diacetyl derivative was prepared and oxidation of (**136**) with potassium permanganate yielded guanidine and the pyrrololactam (**137**), which was reduced to the alcohol with sodium borohydride[124].

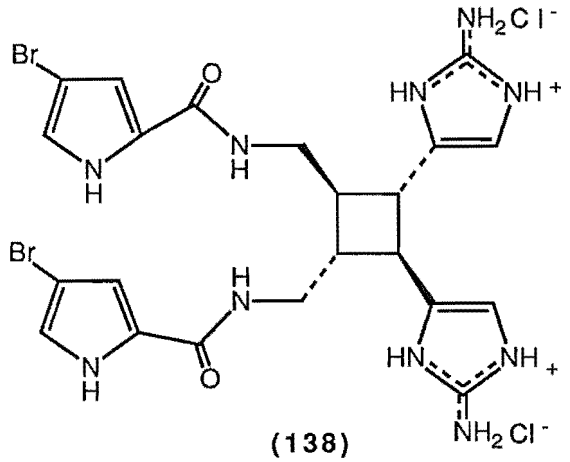


(**136**)



(**137**)

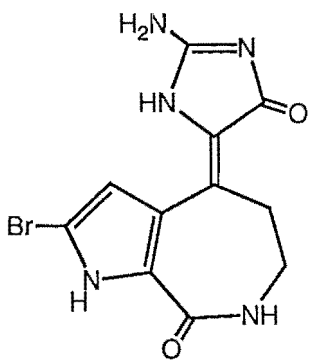
Faulkner *et al*. isolated sceptrin (**138**), the monobromo dimer of oroidin, from *Agelas sceptrum*, along with oroidin itself. Sceptrin was found to be the major antimicrobial constituent of the sponge and an X-ray crystal structure of the compound was obtained[125]. Sceptrin could result from a head-to-head cycloaddition reaction of monobromo-oroidin and since this is an allowed photochemical reaction, a number of photodimerization reactions of oroidin were attempted, but all proved unsuccessful.



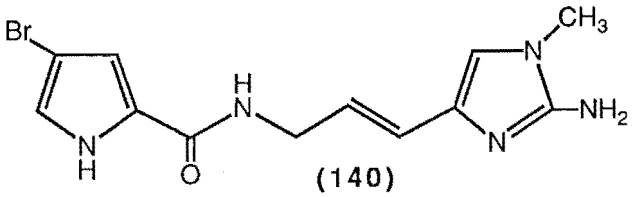
(138)

In 1982 Buchi and Foley reported a biomimetic synthesis of (**124**). This was achieved by synthesis of dihydrooroidin from (+)-citrulline, followed by oxidative cyclization to give racemic dibromophakellin[126].

In 1982 also, Cimino *et al.* reported the isolation of (**139**) from two marine sponges: *Axinella verrucosa* and *Acanthella aurantiaca*. This compound was found to be moderately cytotoxic *in vitro* but inactive *in vivo* against P388 leukaemia cells[127]. An X-ray crystal structure was obtained[128] and the diacetate derivative was prepared. The following year, Kitagawa *et al.* reported compounds (**139**) and (**136**) from *Hymeniacidon aldis*, (Hymeniacidonidae), naming (**139**) hymenialdisine and repeating the X-ray analysis[129].



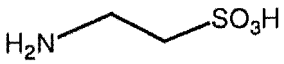
(139)



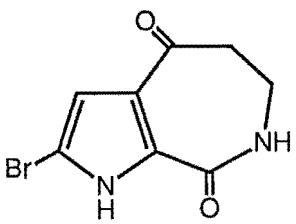
(140)

Keramidine (**140**), an antagonist of serotonergic receptors, was isolated from the Okinawan sponge *Agelas* sp. by Nakamura *et al.* in 1984[130]. This compound is similar to oroidin but is monobrominated in the three position of the pyrrole ring and N-methylated on one of the guanidine nitrogens.

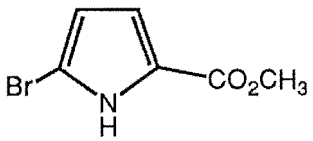
In 1984 Maksimov *et al.* isolated the pyrrololactam (**137**) from the Madagascan sponge *Acanthella carteri*. This compound had previously been prepared[122] but this was the first reported isolation from a natural source. Compounds (**136**) and (**139**) were also isolated, along with the unusual amino acid taurine (**141**)[131]. The same group of workers isolated compound (**137**) from a Tanzanian Axinellid, along with compounds (**136**), (**124**), (**139**) and (**126**)[132].



(**141**)

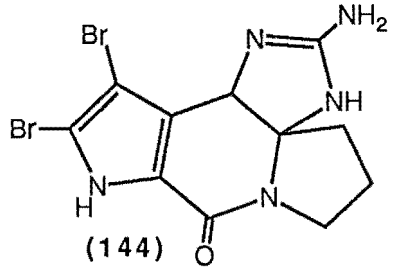


(**142**)

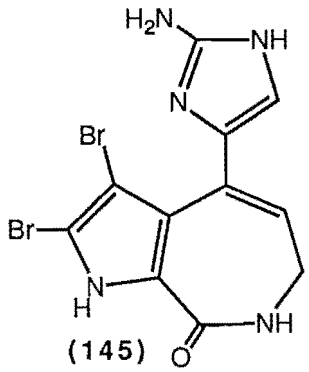


(**143**)

In the same year, Schmitz *et al.* reported the isolation of (**137**) which he called aldisin, and a monobromopyrrololactam (**142**) from *Hymeniacidon aldis* and also from an unidentified Fijian sponge. Compound (**137**) was also isolated from the Fijian sponge, and it is likely that compounds (**137**) and (**142**) are actually degradation products of compounds (**136**) and (**139**). 2-Bromoaldisin, (**142**) was isolated from a Sri Lankan *Lissodendoryx* species, (Myxillidae), Order-Poecilosclerida, along with 5-bromo and 4,5-dibromopyrrole-1-carboxylic acid methyl esters (**143**) and (**129**)[133]. These were probably artefacts of work-up however as found by Minale[116]. Clearly the taxonomy of the so-called *Lissodendoryx* species requires further investigation in light of what is known of the distribution of bromopyrroles among the Porifera.

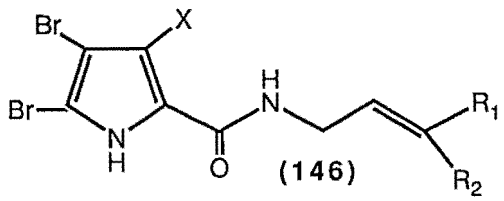


(**144**)

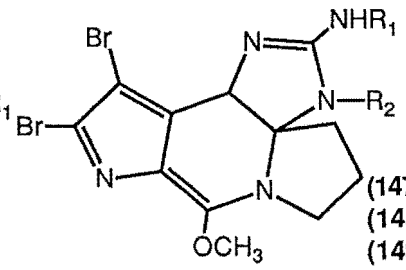


(**145**)

In 1985 also, Ahond and Poupat isolated nine pyrrolic metabolites from the New Caledonian sponge *Pseudaxinyssa cantharella*, (Axinellidae)[134]. These included the new compounds dibromocantharelline (**144**) and odiline (**145**) and oroidin, hymenialdisine, compounds (**137**), (**136**), (**142**) and (+)-dibromophakellin, the enantiomer of (**124**). The structures of (**144**) and (+)-dibromophakellin were confirmed by X-ray analysis. The structure of the most polar metabolite (**146**), was not fully determined. Acetylation, debromination and hydrogenation reactions were carried out on some of these compounds. Dibromocantharelline underwent enolisation in basic media and was then methylated and acetylated to yield compounds (**147-149**). A large quantity of taurine was also isolated from the sponge.



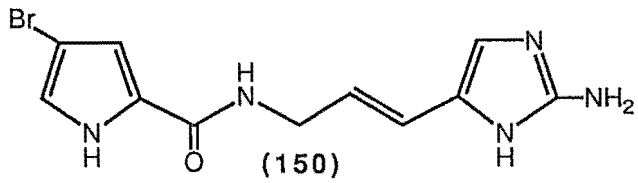
(**146**)



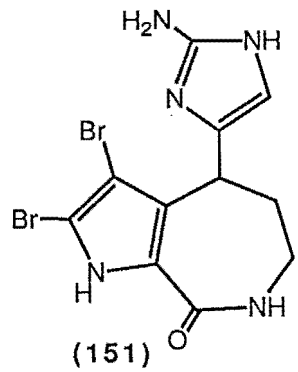
	R1	R2
(**147**)	H	H
(**148**)	$COCH_3$	H
(**149**)	$COCH_3$	$COCH_3$

At around the same time, Faulkner also reported the isolation of (**145**) which he called stevensine, and sceptrin from an unidentified Micronesian sponge[135].

The enantiomer of dibromocantharelline was isolated by Maksimov *et al.* from *Acanthella carteri* in 1986 and an X-ray crystal structure analysis obtained. This was named dibromoisophakellin. Debromination occurred under catalytic hydrogenation conditions[136].



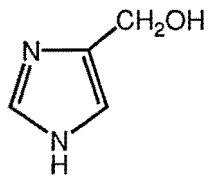
(**150**)



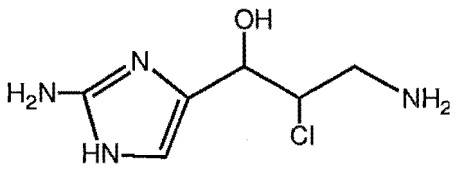
(**151**)

Kobayashi *et al.* isolated two more bromopyrroles from *Hymeniacidon* sp., hymenidin (**150**), the monomer of sceptrin and an antagonist of serotonergic receptors[137] and hymenin (**151**) which was found to be both antibacterial and an α-adrenoceptor blocking agent[138]. Hymenin is thought to arise from an intramolecular cyclization of oroidin.

In 1986 Ahond and Poupat reported a synthesis of oroidin by two different routes, both starting with 4(5)-hydroxymethylimidazole (**152**). Yields in both cases were very low[139].



(152)



(153)

Perhaps the most promising metabolite of this type in terms of biological activity which has been isolated thus far, is girolline (**153**), isolated from *Pseudaxinyssa cantharella* by Ahond and Poupat. This compound is active against P388 leukaemia cells, both *in vitro* and *in vivo*. The bromopyrrole group is absent and girolline contains an unusual chlorohydrin functionality[140]. Girolline could conceivably be formed in the sponge through hydrolysis of oroidin, followed by addition across the double bond to form the chlorohydrin. Again, girolline occurs naturally as a hydrochloride. A total synthesis of girolline has recently been achieved, using 4(5)-hydroxymethylimidazole (**152**) hydrochloride as the starting material, as for the synthesis of oroidin[141]. The *threo*- isomer had the same activity and spectral characteristics as the natural product.

Table 4.1 summarises the bromopyrrole compounds isolated to date, with the sources they were obtained from.

Table 4.1 Summary of Bromopyrrole Compounds.

Structure	Name	Source	Reference	Comment
(124)	dibromophakellin	*Phakellia flabellata*	114	(-)-dibromophakellin.
			115	
			122	Low basicity reported. Oxidation and acid treatment. X-ray of monoacetate
			126	Biomimetic synthesis.
		Tanzanian Axinellid	132	Isolation.
		Pseudaxinyssa cantharella	134	(+)-dibromophakellin. X-ray structure.
(125)	monobromo-phakellin	*Phakellia flabellata*	114	
			115	
			122	
(126)	4,5-dibromo-pyrrole-2-carboxylic acid	*Agelas oroides*	116	Isolation and as a reaction product.
		Tanzanian Axinellid	132	Isolation.
(127)	2-cyano-4,5-dibromopyrrole	*Agelas oroides*	116	Isolation and hydrolysis
(128)	2-amino-4,5-dibromopyrrole	*Agelas oroides*	116	Isolation and as a reaction product.
(129)	4,5-dibromo-pyrrole-2-carboxylic acid methyl ester	*Agelas oroides*	116	Artefact of isolation. procedure.
		Lissodendoryx species	133	
(130)	oroidin	*Agelas oroides*	116	Isolation. Incorrect structure. Derivatisation.
			117	Correct structure. Synthesis of monoacetate.
		Axinella verrucosa	118	Isolation.
		Axinella damicornis		

Table 4.1 continued

		Agelas sceptrum	125	X-ray not reported.
		Pseudaxinyssa cantharella	134	Isolation. Derivatisation.
			139	Synthesis.
(131)		*Agelas* sp.	119	Isolation.
(132)	midpacamide	unidentified sponge	121	Isolation and base hydrolysis.
(133)	1-methyl-4,5-dibromopyrrole -2-carboxylic acid	unidentified sponge	121	Isolation. Product of hydrolysis of **(132)**.
(136)		*Phakellia flabellata*	123	Partial structure.
			124	Full structure. Acetylation and oxidation.
		Hymeniacidon aldis	129	Isolation.
		Acanthella carteri	131	Isolation.
		Tanzanian Axinellid	132	Isolation.
		unidentified sponge	133	Isolation.
		Pseudaxinyssa cantharella	134	Isolation.
(137)			122	Oxidation product of **(125)**.
		Acanthella carteri	131	Isolation.
		Tanzanian Axinellid	132	Isolation.
	aldisin	*Hymeniacidon aldis* and unidentified sponge	133	Isolation.
		Pseudaxinyssa cantharella	134	Isolation.
(138)	sceptrin	*Agelas sceptrum*	125	Isolation and X-ray structure.
		unidentified sponge	135	Isolation.

Table 4.1 continued.

(139)		*Axinella verrucosa*	127	Isolation, X-ray and acetylation.
		Acanthella aurantiaca		
	hymenialdisine	*Hymeniacidon aldis*	129	Isolation and X-ray structure.
		Acanthella carteri	131	Isolation.
		Pseudaxinyssa cantharella	134	Isolation.
(140)	keramidine	*Agelas* sp.	130	Isolation.
(141)	taurine	*Acanthella carteri*	131	Isolation.
		Pseudaxinyssa cantharella	134	Isolation.
(142)	2-bromoaldisin	*H. aldis*	133	Isolation.
		unidentified sponge		
		Lissodendoryx species		
		P. cantharella	134	Isolation.
(143)	5-bromo-carboxylic acid methyl ester	*Lissodendoryx* species	133	Artefact.
(144)	dibromo-cantharelline	*P. cantharella*	134	Isolation, X-ray structure and reactions.
	dibromo-isophakellin	*A. carteri*	136	Isolation and X-ray structure. Enantiomer of **(145)**.
(145)	odiline	*P. cantharella*	134	Isolation.
	stevensine	unidentified sponge	135	Isolation.
(146)		*P. cantharella*	134	Structure not fully determined.
(150)	hymenidin	*Hymeniacidon* species	137	Isolation.
(151)	hymenin	*Hymeniacidon* species	138	Isolation.
153)	girolline	*P. cantharella*	140	Isolation and derivatisation.
			141	Synthesis.

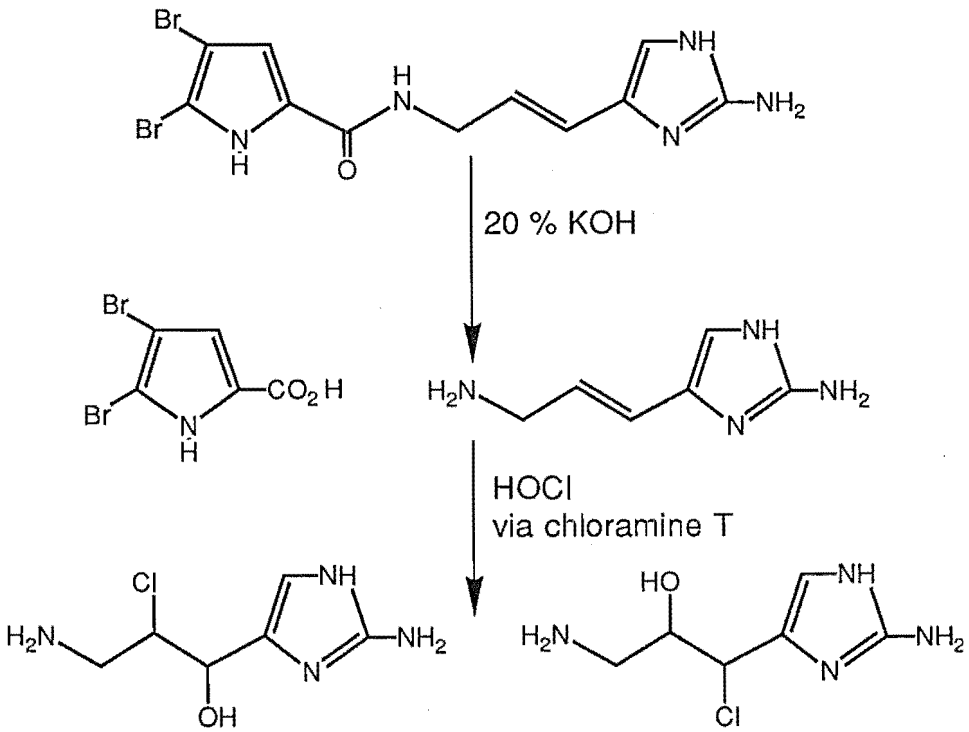
4.2 STUDIES OF THE SPONGE *AXINELLA* SP. 2

INTRODUCTION

A crude extract of the sponge, *Axinella* sp. 2 in the collection of the University of Canterbury Marine Chemistry group, exhibited strong cytotoxicity in the P388 assay and strong antiviral activity in the antiviral/cytotoxicity assay. Preliminary work on an extract of this sponge by a member of the group, Dr. Nigel Perry, indicated that the compound responsible for this activity was probably girolline (**153**), previously isolated from the New Caledonian sponge, *Pseudaxinyssa cantharella*[140]. Dr. Perry also noted the presence of oroidin (**130**), taurine (**141**) and some ring-contracted norsterols in the sponge extract.

Further work on the sponge was undertaken for several reasons. It was considered necessary to prove that girolline was the compound responsible for the observed biological activity, so isolation and purification of this compound had to be achieved. Also, if sufficient quantity of oroidin could be isolated from the sponge extract, it was hoped to carry out reactions of this compound as shown in Scheme 4.1 to synthesise girolline and its structural and stereoisomers and to assay them for biological activity.

Scheme 4.1: Proposed reaction scheme for the conversion of oroidin to girolline.



The first of these objectives proved to be a difficult task in itself, due to the problems of separating small water soluble molecules from other similarly sized compounds and salts. However, a combination of reverse phase column chromatography and gel permeation chromatography led to the isolation of girolline as the antiviral and major cytotoxic component of the extract.

In addition to girolline, the known compound hymenialdisine was isolated as a major cytotoxic sponge component and an X-ray crystal structure analysis performed. Several discrepancies between the experimental data obtained on hymenialdisine and the published data were noted.

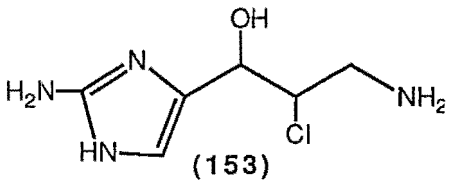
The quantity of oroidin in the sponge extract was not as great as first supposed and the poor solubility of this compound rendered it difficult to work with. A tlc survey of all other sponge extracts of the family Axinellidae in the Marine Chemistry group's collection was undertaken, in an effort to find other sponges containing oroidin which could be extracted to gain sufficient compound for the proposed reaction chemistry to be performed. Unfortunately, oroidin was not detected in any other extracts.

The sterol composition of *Axinella* sp. 2 was found to consist exclusively of A-norstanols, a feature characteristic of many Axinellidae sponges.

ISOLATION OF GIROLLINE (**153**)

Reverse phase flash column chromatography of the crude sponge extract, led to the antiviral activity being concentrated in the first three fractions from the column. These were combined and partially desalted by dissolving in methanol/water (4:1) and filtering. Gel permeation chromatography of the methanol soluble material led to the biological activity being retained. Subsequent elution with 0.25 M acetic acid yielded crude girolline acetate which was converted to girolline hydrochloride by the addition of concentrated hydrochloric acid. The sample was again desalted by dissolving it in methanol and filtering. The methanol soluble material was subjected to reverse phase hplc to yield a fraction, the ^{1}H nmr spectrum of which, contained only signals arising from girolline (**153**). Elemental analysis of this fraction revealed only 1.5% carbon, indicating the presence of a large amount of inorganic salts. Further gel permeation chromatography led to the isolation of girolline as an opaque oil, again pure by nmr analysis. Elemental analysis now revealed 10% carbon, which, with the theoretical value being 27.4%, indicated that

attempts to remove the inorganic salts had not been entirely successful. However, as this was not a new compound and had already been extensively studied by another group of researchers[140,141] and the amount of material that would be obtained from further purification would not be sufficient to examine the reaction chemistry of girolline, efforts to remove the remaining inorganic material were discontinued.

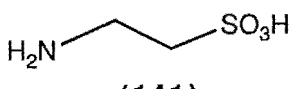


(153)

The ^1H nmr spectrum of girolline in D_2O was consistent with the published data, as was the ^{13}C nmr spectrum[140]. The FAB mass spectrum showed ions at 367 $[M + matrix + Na]^{+\bullet}$ and 331 $[M + matrix + Na - HCl]^{+\bullet}$. The ir spectrum of the isolated girolline contained O-H and N-H stretching bands at 3500 and 3310 cm^{-1} and a strong C-Cl stretching band at 610 cm^{-1}. The ORD curve of girolline was negative, confirming that the stereochemistry of the isolated compound is identical to that of the previously reported compound. The isolated girolline had an IC_{50} of 100 ng/ml in the P388 assay and showed antiviral activity against both *Polio* and *Herpes* viruses at a doseage of 0.5 µg per disc, with cytotoxicity type C7*. As the elemental analysis results would seem to indicate that the isolated girolline is only 33% pure by mass, the activity of the pure compound is probably much higher. The antiviral activity of girolline has not previously been reported. Girolline shows significant antitumour activity against both human and murine cell lines and is in Phase 1 of clinical study[142].

TAURINE (**141**)

A fraction from the first gel permeation column of the water soluble partition of the extract was shown to contain a major component by ^1H and ^{13}C nmr spectroscopy in D_2O. On standing, a white precipitate settled out, the ^1H and ^{13}C nmr spectra of which were consistent with those reported for taurine[143,144].



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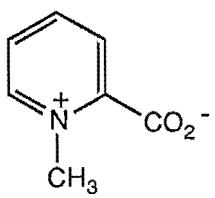


(94)

HOMARINE (**94**)

Fractions from the gel permeation columns of the water soluble material from the extract were analysed by ^1H nmr spectroscopy in D_2O. Homarine was identified in several fractions by comparison of the ^{13}C nmr data with literature values[78].

ISOLATION OF HYMENIALDISINE (**139**)

A later fraction from the initial chromatography column on the extract showed antimicrobial activity against *Bacillus subtilis*. Over time a pale yellow powder settled out of this fraction. Recrystallisation from methanol yielded a small quantity of good quality cubic crystals. A better yield was obtained by recrystallisation from aqueous methanol using trifluoroacetic acid fumes to aid dissolution and triethanolamine fumes to neutralise the solution, to give pale yellow needles.

The crystals were insoluble in chloroform, but a ^1H nmr spectrum of a sub-sample of these crystals was obtained in deuterated chloroform/trifluoroacetic acid (9:1). The spectrum (Table 4.2) contained two sharp two proton multiplets at 3.59 and 3.61 ppm, a sharp one proton doublet (2.6 Hz) at 6.57 ppm and three broad singlets at 8.18, 9.2 and 10.49 ppm, which each integrated as one proton.

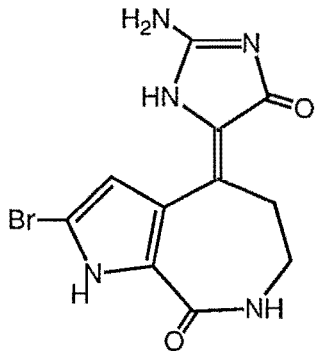
Table 4.2: ^1H nmr data for Hymenialdisine (**139**)

POSITION	$CDCl_3$/TFA (9:1)	DMSO-d6	DMSO-d6 + TFA or HCl
1	10.49 br s	12.95 br s	13.0 br s
3	6.57 d (2.6)	6.71 s	6.60 d (2.3)
7	8.18 br s	8.19 br s	8.21 br s
8	3.59 m[a]	3.37 br s	3.36 s
9	3.61 m[a]	3.37 br s	3.36 s
15	9.2 br s	8.8 br s	8.9 br s*
14'	not seen	not seen	9.6 br s*

[a]Assignments as per reference 133
*Assignments in vertical columns may be interchanged.

The ^1H nmr spectrum obtained in DMSO-d6 was less well defined, with a broad four proton singlet at 3.37 ppm, one proton singlets at 6.71, 8.19, and 12.95 ppm and a very broad singlet at 8.8 ppm. The addition of a drop of trifluoroacetic acid

to the sample moved the signal at 6.71 ppm to 6.60 ppm and sharpened it to a doublet (2.3 Hz) and another broad singlet at 9.6 ppm was evident. A ^{13}C nmr spectrum (Table 4.3) was obtained in this solvent system. Ten carbon resonances were evident, with that at 32.41 ppm being of low intensity.



(139)

Table 4.3: ^{13}C nmr data for Hymenialdisine (**139**)

CARBON	DMSO-d6/TFA	DMSO-d6/HCl	DMSO-d6
2	105.12	106.23	103.48
3	111.24	112.05	115.36
4	121.60*	122.29*	124.16*
5	128.51	128.65	126.90*
6	162.44	163.08*	162.60
8	solvent obscured	39.39	solvent obscured
9	32.41	32.89	29.42
10	128.80	129.50	126.32*
11	121.22*	121.17*	124.10*
12	163.44	163.41*	171.65
14	154.52	154.95	155.97

*Assignments in vertical columns may be interchanged.

A HETCOR nmr experiment showed only three correlations. The proton resonance at 6.60 ppm was correlated to a carbon resonance at 111.24 ppm and the proton resonance at 3.36 ppm showed correlations to the carbon resonance at 32.41 ppm and the solvent peak. The latter correlation was at first assumed not to be real, but was later realised to be a true correlation to a carbon resonance, obscured by the solvent signal.

At this stage, the literature was checked for other compounds isolated from the Axinellida. The data was most consistent with that published for hymenialdisine

(**139**), first isolated by Cimino *et al.* from *Axinella verrucosa* and *Acanthella aurantiaca*[127] and later by Kitagawa *et al.* from *Hymeniacidon aldis*[129]. The experimental nmr data however was not entirely consistent with that reported by either of these two groups of workers, with the most notable differences being for the imidazole ring. A negative ion DCI mass spectrum obtained using methane as reagent gas, clearly showed an appropriate isotope pattern for one bromine atom and established the molecular formula as $C_{11}H_{10}BrN_5O_2$, with parent ions at 325/323, as for hymenialdisine. The compound decomposed at 220°C (turning a deep red), while the reported melting point for hymenialdisine was 160-164°C (decomposition)[129]. Uv and ir spectroscopic data obtained were similar to reported values but not identical.

A single crystal X-ray structure analysis was performed to attempt to clarify the discrepancies between published and observed data. The study established that the compound was indeed hymenialdisine. As for the two previously published X-ray crystal structure analyses of this compound[128,129], the structure was solved by the heavy atom method and a molecule of methanol was incorporated into the crystal lattice per molecule of hymenialdisine. No significant differences were observed between the X-ray data and that previously reported. Figure 4.3 shows the structure of hymenialdisine as determined by single crystal X-ray analysis.

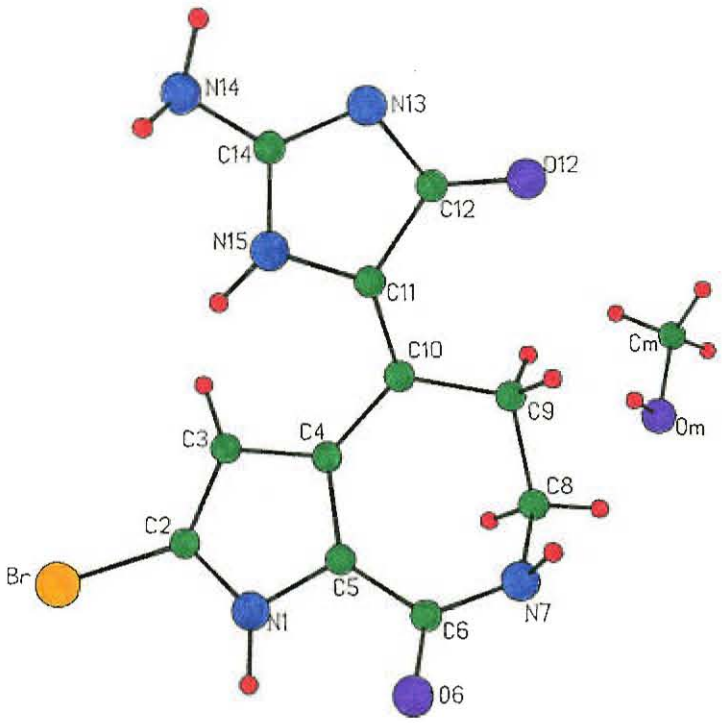


Figure 4.3: The X-ray crystal structure of hymenialdisine (**139**).

This did not explain the differences in nmr data or melting point. As the differences probably arose from the previously reported data being recorded for the free base and the experimental data for the protonated form, the sample from which the crystals for the X-ray analysis were selected was dissolved in DMSO-d6 with a drop of TFA added and the ^{1}H and ^{13}C nmr spectra recorded. The spectra were identical to those obtained prior to the X-ray analysis and the crystals used in the X-ray study again decomposed at 215-220°C. The same sample was then flushed down a reverse phase column to remove the DMSO and the ^{1}H and ^{13}C nmr spectra of the sample obtained in just DMSO-d6. The spectra were this time in accord with those reported by Cimino[127], although the assignments of C6 and C14 were reversed (Table 4.3). The nmr data reported by Kitagawa was obtained in DMSO-d6 with two drops of 2M hydrochloric acid added[129]. This experiment was repeated and the data did not agree with that of Kitagawa. Not surprisingly, it was consistent with that of the protonated form (Table 4.3).

Table 4.4: HETCOR, XCORFE and HMBC correlations observed for hymenialdisine (**139**) in DMSO-d6/TFA.

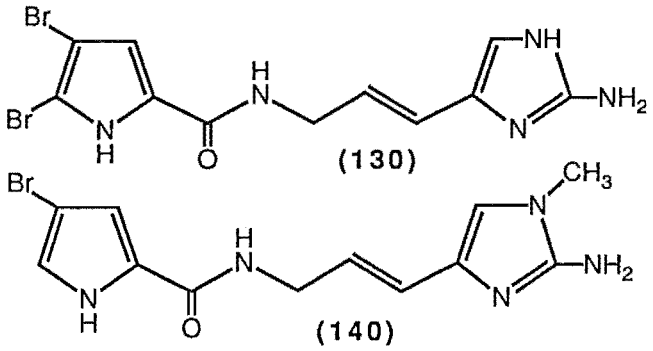
HETCOR	H3	<->	$C_{111.24}$	XCORFE	H3	<->	$C_{105.12}$
	H8, 9	<->	$C_{32.41}$			<->	$C_{121.60}$
		<->	solvent			<->	$C_{128.51}$
					H8, 9	<->	$C_{121.22}$
HMBC	H1	<->	$C_{121.22}$			<->	$C_{121.60}$
	OR	<->	$C_{121.60}$			<->	$C_{128.80}$

The ^{13}C nmr spectrum of the protonated form (Table 4.3) was assigned with the aid of XCORFE and HMBC nmr experiments (Table 4.4) and by reference to published data for the protonated form of the debromo compound (**136**)[124]. An XCORFE nmr experiment showed correlations from H8, 9 to carbon signals at 121.22, 121.60 and 128.80 ppm. The signal at 6.60 ppm assigned to H3, showed correlations to carbon resonances at 121.60 and 128.51 ppm. The correlation to the resonance at 128.51 ppm was a three bond correlation, no matter whether the resonance at 128.51 ppm arose from C5 or C10. If this resonance arose from C5 however, correlations from H8, 9 to it would be over four bonds and no such correlations were observed. The observed correlation from H8, 9 was to the carbon signal resonating at 128.80 ppm and if this was assigned as C10, these correlations

would be over two and three bonds, which is more likely. The signal at 128.51 ppm was therefore assigned as C5 and that at 128.80 ppm assigned as C10. H3 showed a correlation by XCORFE to a carbon signal resonating at 121.60 ppm, which would be a two bond correlation if this signal was assigned as C4 and a four bond correlation if assigned as C11. An HMBC nmr experiment on hymenialdisine revealed one further correlation. The proton signal at 13.0 ppm assigned to H1, showed a correlation to either the carbon resonance at 121.22 ppm or that at 121.60 ppm, but it could not be determined to which. As it is probably more likely that the observed XCORFE correlation from H3 would be over two rather than four bonds, the signal at 121.22 ppm was assigned as C11 and that at 121.60 ppm as C4. These assignments however, could be interconverted (Table 4.3). The ^{13}C nmr spectrum of hymenialdisine in DMSO-d6/HCl was assigned by analogy to that obtained in DMSO-d6/TFA, while that obtained in DMSO-d6 alone was assigned in a similar manner, and by reference to previously published assignments for hymenialdisine and compound (**136**)[124,129,133].

A situation similar to this is reported in the literature for the debrominated compound[133]. A large difference in melting point from previously published data was observed, so the free base of the compound was prepared and melting point, nmr and mass spectra obtained. It is interesting to note that in this case, "The nmr spectra were virtually the same as those obtained" for the protonated form[133]. This was not so in the present case. Since discrepancies such as those discussed here can be confusing or misleading to other researchers, besides sometimes leading to inaccuracies in the literature, it is obviously important when dealing with compounds of this type to determine if one has a free base or a salt, and to carefully monitor and record the experimental conditions used when measuring the physical properties of such a compound.

Cimino found hymenialdisine to be "moderately cytotoxic" *in vitro*[127]. In our assay systems, hymenialdisine was found to show cytotoxicity of type C11 in the antiviral/cytotoxicity assay and a 1 mm inhibitory zone against *Bacillus subtilis* at 200 μg per disc in the antimicrobial assay. The IC_{50} of hymenialdisine was determined to be 700 ng/ml in the P388 assay.



OROIDIN (**130**)

Dr. Perry found that a ^{13}C nmr spectrum in DMSO-d6 of fraction 5 from the initial chromatography column of the extract revealed the presence of oroidin (**130**), the data corresponding well with literature values[134]. Dr. Perry was also able to assign the ^{1}H nmr spectrum in DMSO by comparison with that published for keramidine (**140**)[130]. Using a solvent system of ethyl acetate, acetone, formic acid and water, known to put oroidin at an Rf of approximately 0.7[134], Dr. Perry analysed the fractions from the column and detected oroidin as weak spots under ultraviolet light in fractions four to seven. A small sample of oroidin was purified and the ^{1}H and ^{13}C nmr spectra obtained in DMSO-d6. The previous assignment was found to be in error, and the spectra were reassigned with the aid of difference decoupling and HETCOR nmr experiments. Dr. Perry ceased work on the extract at this stage.

After a period of time, the column fractions were examined by ^{1}H and ^{13}C nmr spectroscopy in DMSO-d6, to check for the presence of oroidin. No signals characteristic of this compound were present and it was decided to re-extract the sponge.

A further extraction of *Axinella* sp. 2 was carried out and reverse phase flash column chromatography performed as previously. Tlc and nmr spectroscopic analysis showed fraction seven to contain oroidin. Isolation of oroidin was attempted by various combinations of medium pressure and high pressure liquid chromatography. This resulted in the isolation of only 3 mg of oroidin.

Many problems were encountered in working with this compound. Its low solubility made separation from other components difficult, as rather than be eluted cleanly from a column, it tended to leach off slowly throughout the course of the isolation or adhere to the column. Various amounts of DMSO were added to samples in an attempt to overcome this problem, but the difficulty of removing this solvent from fractions for analysis presented a further problem.

Also, the quantity of oroidin present in the sponge extract was not as great as first supposed, so utilising it to synthesise girolline was no longer feasible. As *Axinella* sp. 2 is a very rare sponge, only known from the sponge garden at Goat Island, Leigh[64], the acquisition of a large collection of sponge was also impractical. It was at this stage that it was decided to survey all other samples of the order Axinellida in the Marine Chemistry group's collection to try and find other Porifera species rich in oroidin.

TLC SURVEY OF AXINELLIDA

All other samples in the collection identified as belonging to the order Axinellida were screened by tlc to check for the presence of oroidin. Where more than one extract of the same species existed, the sample most recently extracted was used. Crude methanol/toluene extracts which had been stored frozen, were spotted onto silica gel plates at very high loadings. The solvent system used previously was employed and samples of crude *Axinella* sp. 2 extract and pure hymenialdisine and oroidin were used as references. Plates were examined under ultraviolet light and by dipping in Sakaguchi reagent[145].

The samples surveyed and the initial biological assay results for each are summarised in Table 4.5. Of the thirty six extracts surveyed, twenty seven showed any detectable biological activity. Of these active extracts, only two exhibited any antimicrobial activity and this was against *B. subtilis*. Twenty three of the twenty seven active extracts showed some antiviral/cytotoxic activity and eleven extracts showed this type of activity only. A variety of cytotoxicity types was observed. Fifteen extracts exhibited some P388 activity and in ten of these, the activity was strong (3+ or 4+). Four extracts exhibited P388 activity only. The only extract with activity in all three assay systems was that of *Axinella* sp. 2, although those of *Axinella* sp. 3, *Axinella* sp., *Pseudaxinella* sp. 1, *Raspailia agminata* and *Raspailia* sp. 5 all showed strong activity in both the antiviral/cytotoxicity and P388 assay systems. No common spots were seen in the active extracts, except those of *Raspailia* sp. 5. Oroidin was not detected in any of the extracts, except that of *Axinella* sp. 2.

Table 4.5: Axinellida samples surveyed by thin later chromatography.

		HSV1	PV1	CYT.	TYPE	P388	AM
Acanthoclada prostrata	(84L02-30)	-	-	-		ND	ND
Acanthoclada sp.	(86K05-02)	2+	2+	-		ND	-
Axinella sp. A	(86L01-04)	?	?	4+	C1	-	-
Axinella sp. 1	(87PH01-02)	-	-	-		-	-
Axinella sp. 2	(86L01-02)	?	?	4+	C1	4+	BS1
Axinella sp. 3	(87MA02-17)	4+	?	+	C7	4+	-
Axinella sp. 4	(83T417-06)	±	±	±		ND	ND
Axinella tricalyciformis	(87GS01-05)	?	?	+	C4	-	BS1
Axinella sp.	(87MA03-11)	-	-	-		1+	-
Axinella sp.	(87MS02-02)	?	?	2+	C6	4+	-
Axinella sp.	(87SI03-03)	-	-	-		-	-
Eurypon sp.	(85K02-15)	?	?	3+	C4	-	-
Eurypon sp. 1	(831129-27)	?	?	3+	C4	ND	ND
Flame Axinellid	(87K12-02)	-	-	±	C6	-	ND
Flame Axinellid	(87MS03-03)	-	-	-		3+	-
Homaxinella sp. 2	(87ANT01-06)	-	-	-		-	-
Homaxinella sp. 3	(87ANT04-02)	-	-	-		-	-
Homaxinella sp.	(85NP03-07)	-	-	3+	C2	ND	ND
Homaxinella sp.	(87ANT01-04)	-	-	-		1+	-
Pararhaphoxya pulchra	(87SI02-03)	-	-	-		-	-
Phakellia dendyi	(86L01-05)	?	?	2+	C1	ND	ND
Phakellia sp. 1	(831130-38)	?	?	±	C6	ND	ND
Pseudaxinella australis	(87SI02-04)	-	-	-		-	-
Pseudaxinella sp. 1	(87SE02-05)	?	?	4+	C11	4+	
Pseudaxinella sp.	(85NP01-09)	-	-	-		ND	ND
Raspailia agminata	(87CR01-05)	?	?	4+	C11	4+	-
Raspailia topsenti	(87CR01-07)	?	?	3+	C4	4+	ND
Raspailia topsenti	(87TP01-05)	?	?	+	C3	1+	-
Raspailia sp. 2	(87FR01-12)	-	-	-		-	-
Raspailia sp. 2 (flat)	(87SI01-17)	-	-	±	C11	-	ND
Raspailia sp. 3	(87SI02-05))	-	-	+	C7	1+	-
Raspailia sp. 5	(87MS03-13)	?	?	4+	C3	3+	-
Raspailia sp. 5	(87MS02-04)	?	?	4+	C11	4+	-
Raspailia sp.	(87BG03-05)	-	-	-		3+	-
Raspailia sp.	(87K11-05)	?	?	2+	C4	ND	ND
Raspailia sp.	(87TS01-15)	?	?	2+	C3	1+	-

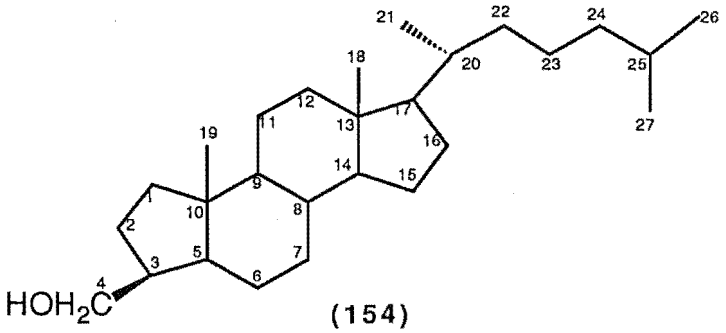
For an explanation of the assay systems used, see Section 1.2, p. 7.

A compound of identical Rf to hymenialdisine was detected in both extracts of *Raspailia* sp. 5. The biological assay results of both extracts were also consistent with the presence of this compound, with strong cytotoxicity of type C11 in the antiviral/cytotoxicity assay and strong P388 activity also. Unfortunately there was no more supply of sponge so it was decided to attempt to identify the compound definitively by using the screening method outlined in Chapter Seven of this thesis on sub-samples of the crude reference extracts. Samples were made up for assay in the three biological assay systems and small scale columns, employing C18 reverse phase and phenyl reverse phase materials and silica gel were run as outlined in Chapter Seven of this thesis. The results are summarised in Figure 7.11 of Chapter Seven, p. 144. These results are consistent with those expected for hymenialdisine, (see Chapter Seven), with the majority of the biological activity concentrated in the final fraction of each column.

The fractions thought to contain hymenialdisine, namely fraction 3 from the C18 and phenyl reverse phase columns and fraction 4 from the silica column, were examined by ^{1}H nmr spectroscopy in $CDCl_3$/TFA (9:1). No signals characteristic of hymenialdisine were observed. This could be due to the level of hymenialdisine being too low to be seen by nmr spectroscopy, but the P388 assay results indicate that if the biologically active component is hymenialdisine, resonances arising from it should be visible in the ^{1}H nmr spectra of the column fractions. Therefore it is likely that in spite of the similar biological activity, chromatographic profile and Rf on tlc, the biologically active component of *Raspailia* sp. 5 is not hymenialdisine. A bulk extraction of sponge would have confirmed this but at the time of writing, no bulk extract was available.

STEROLS

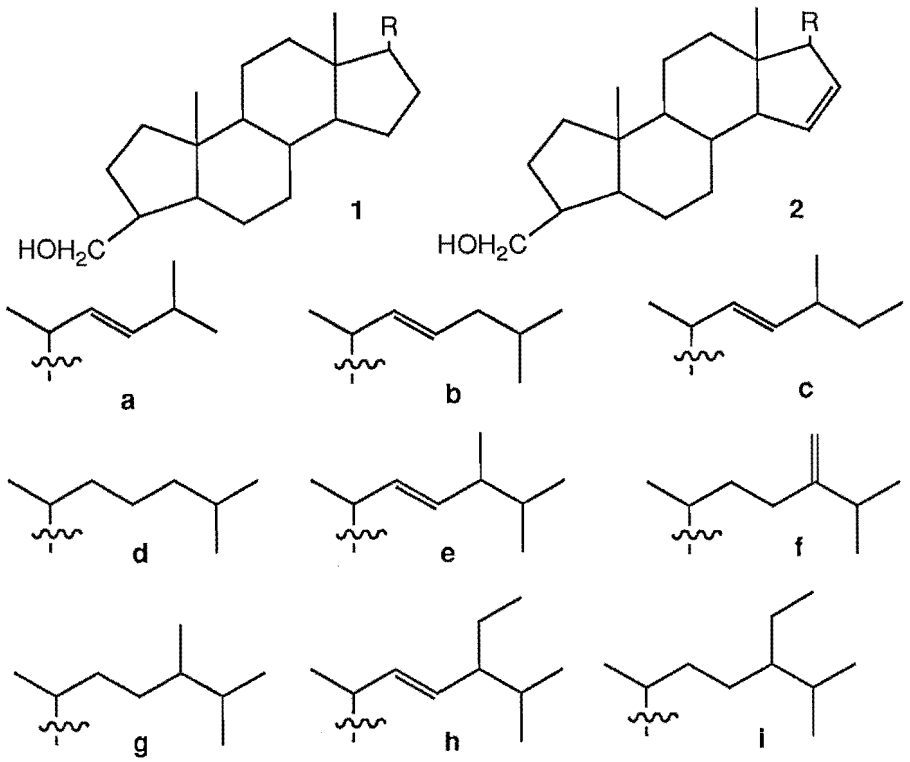
Dr. Perry had examined the sterol composition of *Axinella* sp. 2 and found it to consist mainly of the ring-contracted A-norstanols. He identified the major sponge sterol as the fully saturated sterol, 3β-(hydroxymethyl)-A-nor-5α-cholestane (**154**) by ^{13}C nmr spectroscopy[146].



(154)

The sterol mixture from the second extraction of *Axinella* sp. 2 was isolated by column chromatography on silica gel. Both the ^{1}H and ^{13}C nmr spectra were consistent with (**154**) being the major sterol. Characteristic signals in the ^{1}H nmr spectrum in $CDCl_3$ were two one proton doublets of doublets at 3.46 ppm (10.3, 10.3 Hz), and 3.71 ppm (10.3, 6.3 Hz) for the C4 protons[146].

The sterol mixture was sent to Professor Carl Djerassi for analysis by gcms. Results of this analysis are summarised in Table 4.6 and show that the entire sterol composition of the sponge consists of ring-contracted A norstanols.



(154) 1d	(156) 1c	(158) 1f	(160) 1h	(162)1l
(155) 1a	(157) 1b	(159) 1e	(161) 1g	(163) 2d

Table 4.6: The sterol composition of *Axinella* sp. 2.

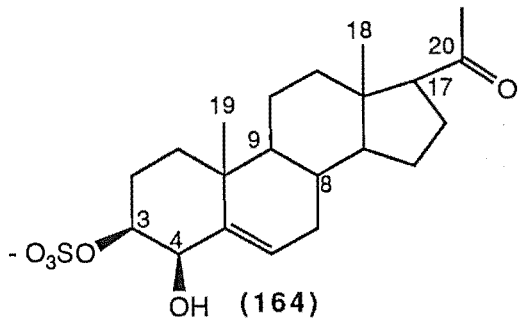
Sterols Present	Percentage composition of the sterol mixture of the *Axinella* sp. 2 sample.
(155)	1
(156)	2
(157)	7
(158)	2
(159)	19
(154)	32
(160)	4
(161)	15
(162)	16
(163)	2

CHAPTER FIVE

5.1 STUDIES OF THE SPONGE, *STYLOPUS AUSTRALIS*

INTRODUCTION

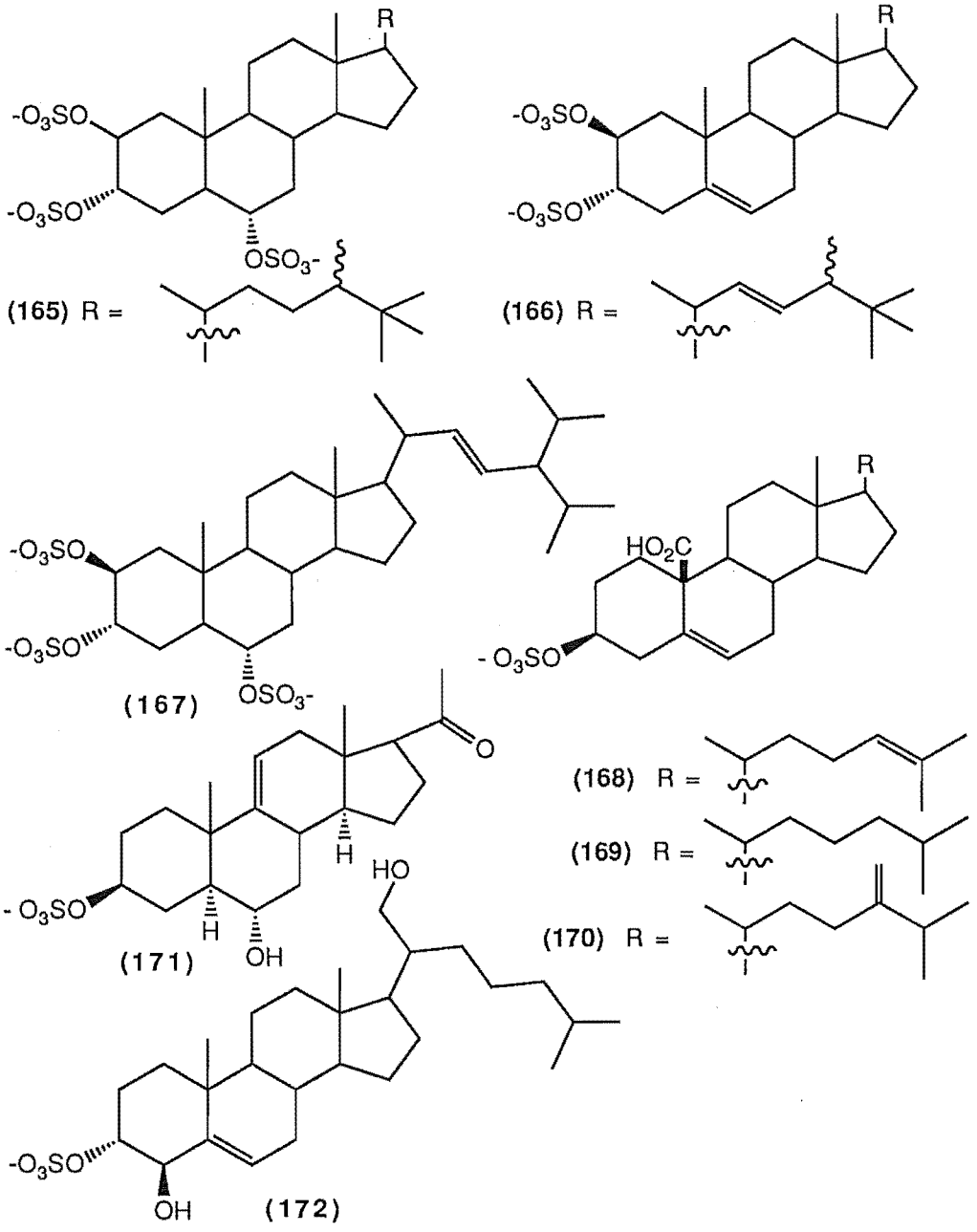
As part of the investigation of extracts from New Zealand marine invertebrates, an extract from the sponge *Stylopus australis* (Hymedesmiidae) was examined. This extract was chosen as it showed biological activity and because a search of the literature revealed that no work had been carried out previously on sponges of this family. It was therefore a possible potential source of novel metabolites. The investigation led to the isolation of the new steroidal sulphate, 3β,4β-dihydroxy-pregn-5-en-20-one-3-sulphate (**164**), a major component of the



(164)

extract. Although the occurrence of steroidal sulphates in a wide variety of echinoderms is well known[147-150,19c], their occurrence in sponges is less well documented. The first report of the isolation of a steroidal sulphate from a sponge was in 1981 when Fusetani *et al.* isolated 24ξ,25-dimethylcholestan-2β,3α,6α-triol-2,3,6-trisulphate (halistanol sulphate) (**165**), as an antimicrobial constituent of the Okinawan sponge, *Halichondria* cf. *moorei* Bergquist[151]. Hemolytic and icthyotoxic activities were also observed for sulphate (**165**). Two similar compounds, (**166**) and (**167**), were subsequently isolated from the sponges *Halichondria* sp. and *Trachyopsis halichondrioides* respectively by a group of Russian workers[152-153,19c]. Three steroidal sulphates (**168-170**), were isolated from the sponge *Toxadocia zumi* by Faulkner *et al.*[154], and these exhibited a range of biological activities, notably strong antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis.* Of the steroidal sulphates isolated to date, those with structural features most similar to (**164**) are 3β,6α-dihydroxy-5α-pregn-9(11)-en-20-one-3-sulphate (**171**), isolated

from the starfish *Asterias amurensis*[147] and cholest-5-en-3α,4β,21-triol-3-sulphate (**172**) isolated from the brittle star *Ophiura sarsi*[149]. The isolation of (**164**) is the first report of a naturally occurring 3β,4β-dioxygenated 5-pregnene.



ISOLATION AND STRUCTURE OF STEROL SULPHATE (**164**)

Frozen *Stylopus australis* sponge was extracted by blending with methanol/toluene and the resulting extract partitioned between ethyl acetate and water. Reverse phase column chromatography of the aqueous phase and subsequent silica gel column chromatography yielded the sterol sulphate (**164**). The

^{1}H nmr spectrum of (**164**) (Figure 5.1) revealed the presence of a double bond proton, two protons attached to oxygenated carbons and three methyl groups, while the ^{13}C nmr spectrum was consistent with the presence of 21 carbon atoms, a single double bond, a ketone functionality and two other oxygenated carbons. These data were suggestive of a steroid nucleus and the structure of (**164**) was further elucidated from comparisons of its ^{13}C nmr chemical shifts (Table 5.1), with those reported for compounds containing the 17β-acetyl function on the one hand, and the Δ^5 3β,4β-diol system on the other hand[81]. A **D**istortionless **E**nhancement by **P**olarisation **T**ransfer (DEPT) nmr experiment was used to distinguish between signals of similar chemical shift but different multiplicity; C7 and C8 for example.

The presence of a sulphate group was suggested by mass spectrometry. The negative ion liquid chromatography (lc) mass spectrum exhibited a molecular ion species at m/z 411 $[M-H]^-$, and fragment ions at 331 $[M-HSO_3]^-$ and 313 $[M-H-H_2SO_4]^-$. The molecular formula for (**164**), $C_{21}H_{32}O_6S$, was obtained from a high resolution fab mass spectrum. Strong ir absorption bands at 1215 and 1255 cm^{-1} supported the presence of a sulphate ester[148].

The stereochemistry of sterol (**164**) was established as 3β,4β from a consideration of H-H coupling constants. Values of 11.1, 3.3 and 3.3 Hz for the H3 coupling constants, corresponding to couplings to the 2β, 2α and 4α protons respectively[155], implied that H3 was in an axial, and therefore α orientation. The observation of large nOe interactions between H4α and H6 and between H3α and H2α and H4α confirmed the stereochemical assignment. The assignment of the ^{1}H nmr spectra of steroids is not a trivial matter, on account of the complex envelope of overlapping signals between 1.2 and 2.2 ppm. The full assignment of the ^{1}H nmr spectrum of (**164**), (Table 5.2), was achieved with the aid of a COSY nmr experiment, selected nOe experiments, HETCOR and XCORFE experiments and comparison of the ^{1}H nmr data of (**164**) with that of other steroids assigned by a combination of the above techniques[156-158].

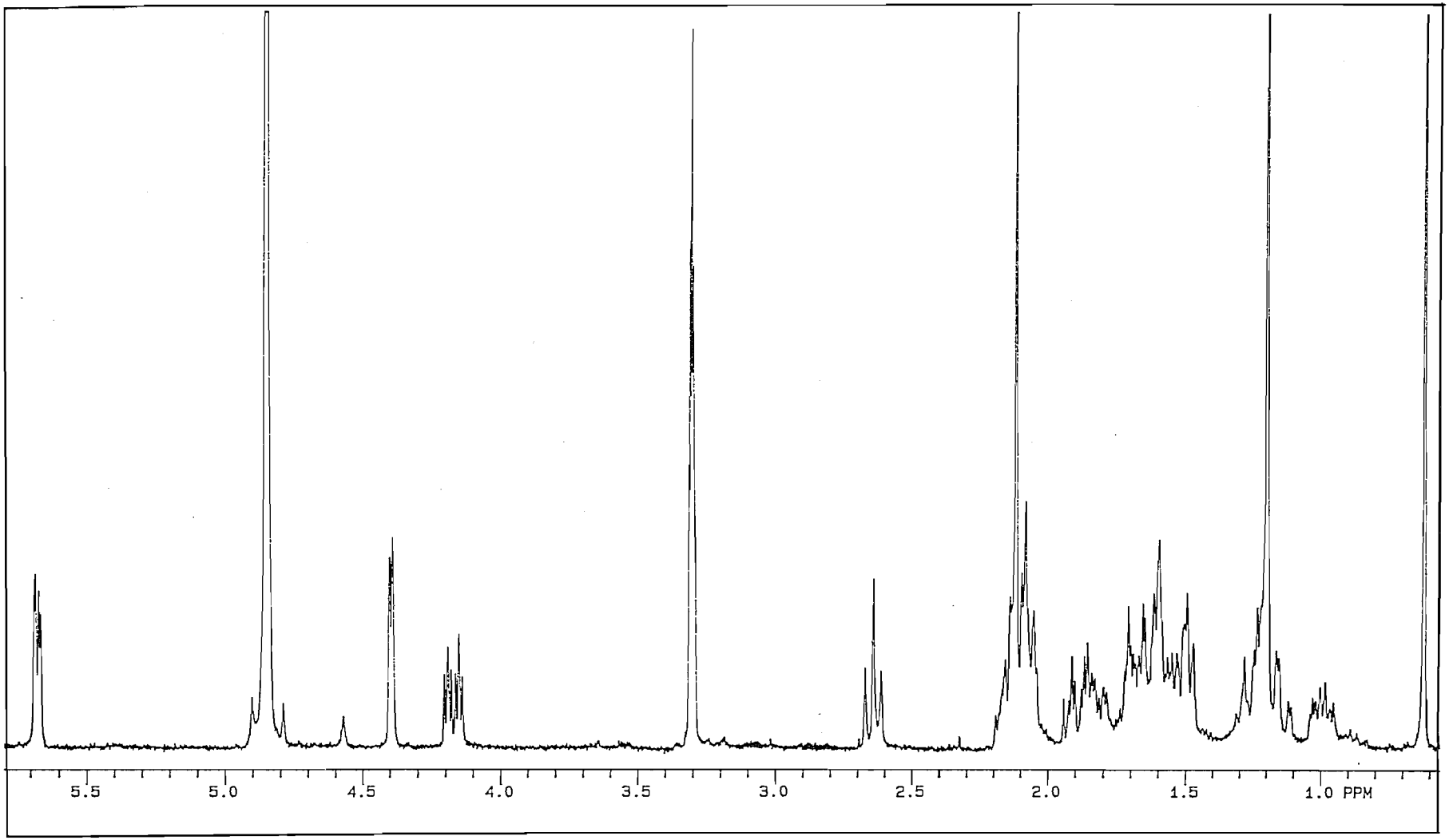


Figure 5.1: The ^{1}H nmr spectrum of compound (**164**) in CD_3OD.

Table 5.1: ^{13}C nmr spectral data for compounds (**164**), (**173**) and (**174**).

	COMPOUND		
CARBON	(**164**)[a]	(**173**)[b]	(**174**)[a]
1	38.98	38.68	39.03
2	25.76	24.44	25.87
3	81.64	77.37	81.68
4	77.15	76.74	77.24
5	143.84	141.67	143.82
6	129.46	126.91	129.66
7	33.45	31.57	33.58
8	33.35	31.61	33.39
9	51.99	50.06	52.34
10	37.65	36.07	37.68
11	21.98	21.75	21.73
12	40.09	36.97	41.02
13	45.42	43.95	43.79
14	58.49	56.90	58.13
15	24.03*	23.56	24.05
16	24.10*	22.83	27.12
17	64.94	63.60	59.63
18	13.88	13.20	12.87
19	21.73	20.51	21.79
20	212.80	209.14	71.16
21	31.98	31.86	24.12
22		170.13	
23		20.56	

[a] Values in ppm for CD_3OD solution.
[b] Values in ppm for $CDCl_3$ solution.
* Assignments in vertical columns may be interchanged.

Table 5.2: ^{1}H nmr spectral data for compounds (**164**), (**173**) and (**174**).

	COMPOUND		
POSITION	(**164**)[a]	(**173**)[b]	(**174**)[a]
1	1.17 α, 1.91 β	1.17 α, 2.08 β	1.17 α, 1.91 β
2	1.79 α, 2.07 β	1.69 α, 2.08 β	1.79 α, 2.07 β
3	4.18 dt (11.1, 3.3, 3.3)	4.43 dt	4.18 dt
4	4.38 d (3.3)	5.72 d (3.0)	4.38 d
6	5.67 dd (5.3, 3.1)	5.81 dd (5.3, 3.4)	5.67
7	1.65 α, 2.10 β	1.65 α, 2.10 β	1.65 α, 2.10 β
8	1.52	1.56	1.52
9	0.98	0.92	0.94
11	1.59 α, 1.47 β	1.59 α, 1.47 β	1.59 α, 1.47 β
12	1.49 α, 2.05 β	1.46 α, 2.08 β	1.49 α, 2.05 β
14	1.15 (3.0)	1.18	1.13
15	1.72 α, 1.26 β	1.62 α, 1.26 β	1.72 α, 1.26 β
16	1.66 α, 2.14 β	1.66 α, 2.07 β	1.66 α, 2.13 β
17	2.64 t (9.0)	2.53 t	2.17 m
18	0.62	0.62	0.77
19	1.20	1.11	1.20
20			3.61 m
21	2.11	2.00	1.09 d (6.1)
23		2.13	

[a] Values in ppm for CD_3OD solution.
[b] Values in ppm for $CDCl_3$ solution.
() coupling constants in Hz.

A summary of the way in which the assignment was achieved is shown in Figure 5.2. There were several signals in the ^1H nmr spectrum of (**164**) which could be unequivocally assigned on the basis of chemical shift and coupling data. These signals were then used as points from which to build up the rest of the assignment. For example, a doublet of doublets in the ^1H nmr spectrum at 5.67 ppm, characteristic of a double bond proton of a Δ^5 steroid, was assigned to H6. Irradiation of this proton signal produced a significant nOe enhancement of a signal at 4.38 ppm which could be assigned to H4α. This signal in turn showed both a COSY correlation to, and nOe enhancement of the signal at 4.18 ppm, allowing assignment of this as H3α. COSY correlations from this proton to signals at 1.79 and 2.07 ppm established these as arising from the protons attached to C2. The observation of an nOe enhancement of the signal at 1.79 ppm on irradiation of the H3α signal determined that this resonance was from H2α and that therefore the 2.07 ppm signal was due to H2β.

In turn, the H2α signal showed a COSY correlation to a signal at 1.17 ppm and a HETCOR correlation from the C1 resonance to a signal at 1.91 ppm established that these two signals were due to the C1 hydrogens. Tables from Schneider et al[157] were used to calculate the effects of various substituents on the chemical shifts of steroid C1 protons. Using the values given for a Δ^5 double bond and a 3β-OH, (assuming the effect of a 3β–SO_4 is similar), chemical shift values of 1.11 ppm for H1α and 1.93 ppm for H1β were calculated. Although no account could be made for the effect of the 4β-OH from these tables, the calculated shifts fit the observed values well, supporting the assignments.

The H6 resonance also showed COSY correlations to signals at 1.65 and 2.10 ppm, in addition to nOe enhancements. These signals therefore must be from H7α and H7β. Calculated values suggested 1.61 ppm for the H7α signal and 1.99 ppm for the H7β signal[157], which allowed assignment of the 1.65 ppm signal as arising from H7α and the 2.10 ppm signal from H7β. Both these proton resonances showed COSY correlations to a signal at 1.52 ppm, establishing this signal as arising from H8, an assignment confirmed by a HETCOR correlation from the C8 resonance. The sequence could be extended further, as this proton resonance was also correlated by the COSY experiment to the signal at 1.15 ppm which was therefore assigned as H14. A HETCOR correlation from the C14 resonance, an XCORFE correlation from the C18 resonance and comparison with the chemical shift of the equivalent proton in progesterone[156] confirmed the assignment.

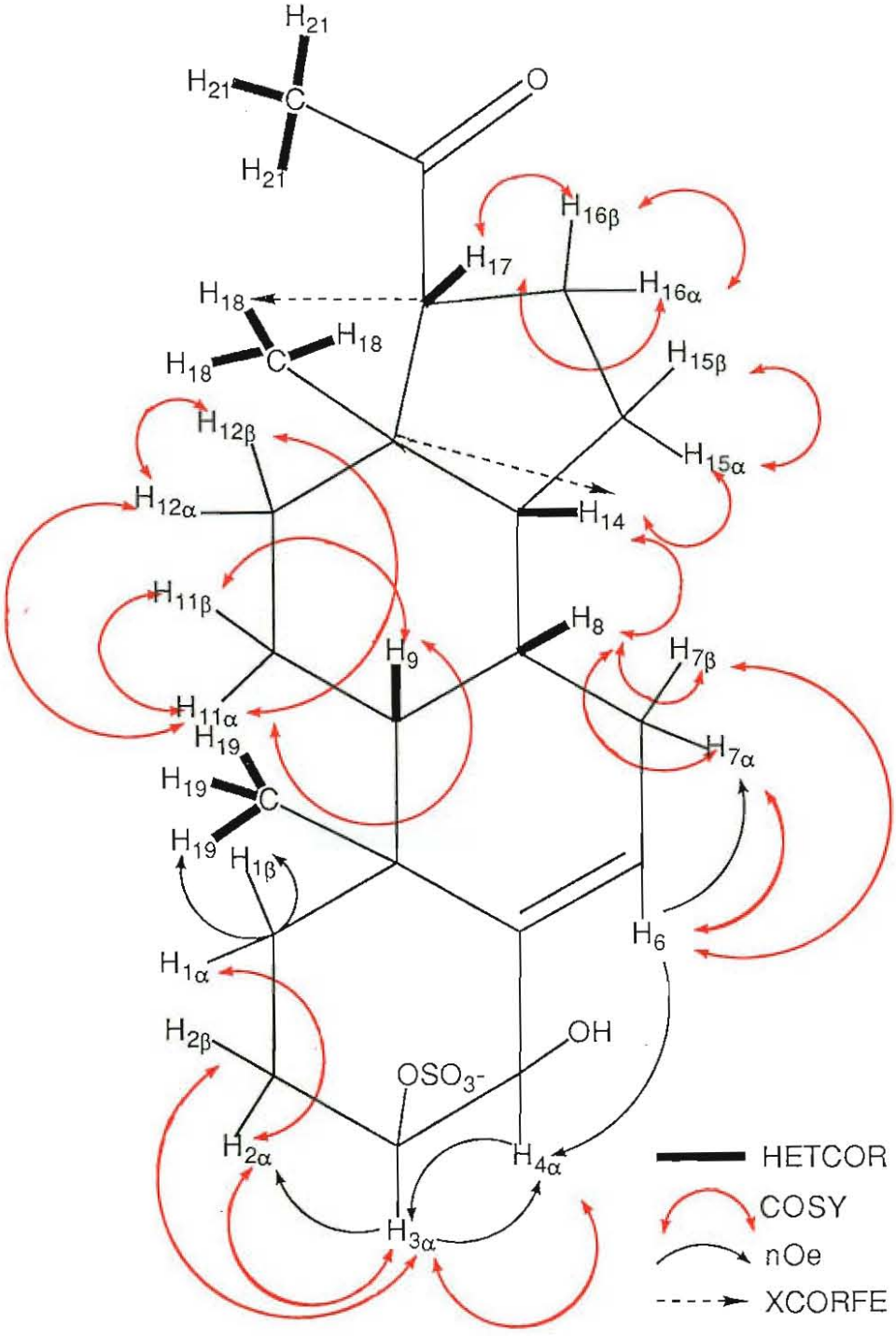


Figure 5.2: nmr correlation diagram for compound (**164**)

The H14 signal showed a COSY correlation to a signal at 1.72 ppm, which must therefore arise from a proton attached to C15. This was in turn correlated to a signal at 1.26 ppm. In progesterone, the H15α signal was assigned as 1.72 ppm and the H15β signal as 1.27 ppm[156], so these assignments are appropriate here.

A signal at 0.62 ppm is established as arising from the H18 protons from its chemical shift and a HETCOR correlation from the C18 resonance. This signal also shows an XCORFE correlation to the C17 resonance, which in turn shows a HETCOR correlation to a signal at 2.64 ppm, assigned to H17.

The H17 signal is correlated to signals at 1.66 and 2.14 ppm from the COSY spectrum, which also shows these signals are correlated to each other. They therefore arise from the protons attached at C16, and comparison with the equivalent proton signals in progesterone established the signal at 1.66 ppm as arising from H16α and that at 2.14 ppm as arising from H16β[156].

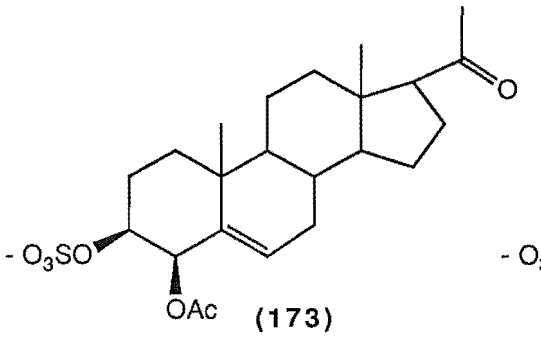
A one proton signal at 0.98 ppm was established as arising from H9 from chemical shift data and the observation of a HETCOR correlation from the C9 resonance. The H9 signal is coupled to signals at 1.59 and 1.47 ppm from a COSY spectrum which showed these were also coupled to each other. Comparison with progesterone data determined the 1.59 ppm signal to be from H11α and the signal at 1.47 ppm to be from H11β. The H11α resonance is coupled to signals at 1.49 and 2.05 ppm, also coupled to each other from a COSY spectrum. Again using progesterone as a model, the 1.49 ppm signal was assigned to H12α and that at 2.05 ppm to H12β[156].

A HETCOR correlation from the C19 resonance to a signal at 1.20 ppm and chemical shift data, established this signal as arising from the H19 methyl protons, as did the observation of an XCORFE correlation to the C1 resonance. Chemical shift data and a HETCOR correlation again were used to assign the H21 methyl protons as the signal at 2.11 ppm, thus completing the assignment of the ^{1}H nmr spectrum.

PREPARATION OF ACETATE (**173**)

Confirmation of the structure was afforded by the acetylation of sterol (**164**) to give the mono-acetylated product (**173**). The ^{1}H and ^{13}C nmr spectra of the acetate (**173**) were consistent with the formation of a monoacetate at position 4. Changes in the ^{1}H nmr spectrum indicative of this were the downfield shifts of the signals for H3, H4 and H6. The H4 resonance exhibited the largest downfield shift,

from 4.38 to 5.72 ppm, while smaller shifts were observed for the H3 and H6 resonances (Table 5.2). A new signal in the spectrum of acetate (**173**) at 2.13 ppm corresponded to the methyl protons of the acetyl group. In the ^{13}C nmr spectrum, two new signals at 170.13 and 20.56 ppm evidenced the formation of the acetate, as did the upfield shift of the C3 signal from 81.64 to 77.37 ppm (Table 5.1). The lc mass spectrum showed m/z 453 $[M-H]^-$ and 395 $[M-C_2H_3O_2]^-$.



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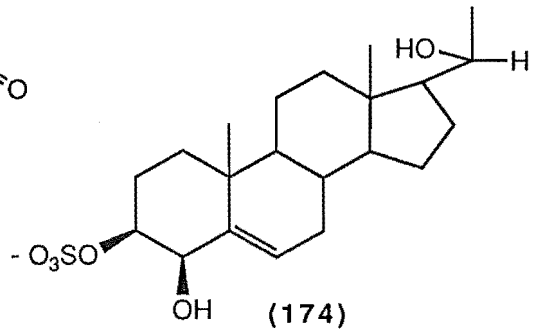

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PREPARATION OF DIOL (**174**)

The pure sterol sulphate was inactive in the antiviral/cytotoxicity, P388 and antimicrobial assay systems. Through preparation of a number of derivatives of the three biologically active sterol sulphates (**168-170**) from the sponge *Toxadocia zumi*, it was found that both the 3β-sulphate and the 19-carboxylic acid functionalities were required for antimicrobial activity[154]. Since (**164**) was inactive in the antimicrobial assay at 200 μg/disc, it was decided to explore the effect on activity, if any, by introducing a more polar group elsewhere in the molecule.

The 3β,4β,20β-trihydroxy-pregn-5-en-3-sulphate (**174**) was prepared by sodium borohydride reduction of sulphate (**164**) in ethanol. After preparative tlc, the major reduction product (**174**) was isolated in good yield.

The ^1H and ^{13}C nmr spectra of (**174**) were similar to those for sterol (**164**) with no changes in the signals in the spectra corresponding to the sulphate region of the molecule. In the ^1H nmr spectrum of (**174**), a new multiplet at 3.61 ppm corresponded to the C20 proton and an upfield shift of the H17 signal from a triplet at 2.63 ppm to a multiplet at 2.17 ppm was observed. The 21-methyl singlet shifted upfield from 2.11 to 1.09 ppm and appeared as a doublet (6.1 Hz) due to coupling to the hydrogen introduced at C20. A slight shift in the H18 methyl signal from 0.62 to 0.77 ppm was apparent (Table 5.2).

The ^{13}C nmr spectrum of (**174**) before preparative tlc showed evidence for formation of both diastereoisomers, with major and minor signals in a ratio of approximately 6:1 for the carbons in the region of the molecule around C20. After preparative tlc however, only the major diastereoisomer was detected. A new signal at 71.16 ppm confirmed the formation of the alcohol at C20, with a corresponding loss of the ketone signal at 212.80 ppm. Other changes in the spectrum were the C17 resonance from 64.94 to 59.63 ppm, the C21 resonance from 31.98 to 24.12 ppm, the C18 resonance from 13.88 to 12.87 ppm and the C16 resonance from 24.10 to 27.12 ppm (Table 5.1).

The stereochemistry of sterol (**174**) was established as 20β on the following evidence. An earlier study of the reduction of 20-ketosteroids, both with sodium borohydride and lithium aluminium hydride, found that the major reduction product was the 20β epimer[159]. For reduction with sodium borohydride, the epimeric ratio of 20β/20α was 7.2:1[159], similar to the approximate 6:1 ratio found here. Further, although the spectra were obtained in different solvents, and in the current work only one epimer was isolated for comparison with literature data, the observed ^{1}H nmr chemical shifts of H18 and H21 for compound (**174**) were more consistent with those reported for 20β-hydroxysteroids than for the 20α epimers[159-161]. Thus, the stereochemistry of compound (**174**) was inferred to be 20β.

The antimicrobial assay of diol (**174**) showed slight activity (<1 mm zone) against *B. subtilis* at 200 μg/disc, indicating that the presence of a polar group at C20 only marginally improves the antimicrobial activity of (**164**). Possibly the mechanism of antimicrobial action of (**168-170**) involves a blocking action through the binding of the molecule to some substrate and with polar groups at C3 and C19, the molecule has the right geometry for efficient binding.

HYDROLYSIS ATTEMPTS ON STEROL SULPHATE (**164**)

All attempts to hydrolyse the sulphate (**164**) to the parent diol were unsuccessful, despite a wide variety of standard methods such as base hydrolysis in pyridine/dioxane and acid hydrolysis in acetic acid being attempted. Either no reaction occurred or complex mixtures of unidentifiable products resulted. Although the presence of a Δ^5 double bond should enhance the rate of solvolysis under acidic conditions, the presence of a 4β-oxygen functionality retards the rate by a similar order of magnitude[162]. If reaction does occur, the conditions required for hydrolysis

of the sulphate lead to elimination from the 4β allylic alcohol, as found for 4β-hydroxy-3β-p-tolylsulphonyloxyandrost-5-en-17-one[162]. Under basic conditions, a complex product mixture was formed, while if hydrolysis of the 20-alcohol (**174**) was attempted, reaction failed to occur.

Reduction of sulphate (**164**) using lithium aluminium hydride gave a mixture of products which was not characterised. None of the products gave a positive sulphate test on tlc with methylene blue dip[163], so it is possible that the products are similar to those formed in the anomalous reduction of 3β-p-tolylsulphonyloxycholest-5-en-4β-ol[164]. A ^{1}H nmr spectrum of the reaction mixture in $CDCl_3$ however, showed no recognisable signals.

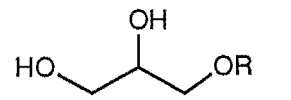
BIOLOGICAL ACTIVITY OF SPONGE

The organic phase of the sponge extract showed strong antiviral activity against a BSC cell line at 100 μg/disc. Partitioning of this phase by normal phase column chromatography on silica gel, led to the activity being concentrated in one fraction from this column. Further column chromatography on silica gel reduced the mass and spread the activity over four fractions, the most active of which only contained 1 mg of material. A comparison of the ^{1}H nmr spectra of these four fractions showed few similarities and indicated that each fraction was probably still a complex mixture. Pursuit of the activity was not continued, due to the extremely low level of the active component and limited supply of the sponge. A sample of crude sponge extract was screened by the procedure outlined in Chapter Seven of this thesis. For the results of this screening, refer to Chapter Seven, p. 144.

GLYCERYL ETHER (CHIMYL ALCOHOL) (**175**)

During the investigation of the active component of the extract, 5 mg of a yellow oil was isolated as one fraction from a silica gel column. A ^{1}H nmr spectrum in $CDCl_3$ of this fraction showed signals at 3.46, 3.50, 3.71 and 3.86 ppm, indicative of those reported for glyceryl ethers[165]. The ^{13}C nmr spectrum of this fraction confirmed this, with signals at 64.33, 70.41, 72.54 and 71.88 ppm for the backbone of the glyceryl ether[165]. This led to the assembly of the basic skeleton of the glyceryl ether (**175**) with the R group being an alkyl chain of unknown length. The length of the chain was determined by mass spectrometry. The CI mass spectrum obtained using NH_3 as reagent gas, was consistent with the molecular formula $C_{19}H_{40}O_3$ and a

molecular weight of 316, showing ions at 334.1987 $[MH + NH_3]^+$ and 317.3051 $[MH]^+$. This allowed assignment of the R group as $C_{16}H_{33}$. That the R group was an unbranched alkyl chain was determined from a DEPT nmr experiment, with the observation of only one methyl group signal in the ^{13}C nmr spectrum. This confirmed that the oil was the known compound, 2,3-dihydroxy-1-hexadecyl-oxypropane or chimyl alcohol (**175**)[165,90].



(175) R= $CH_2(CH_2)_{14}CH_3$

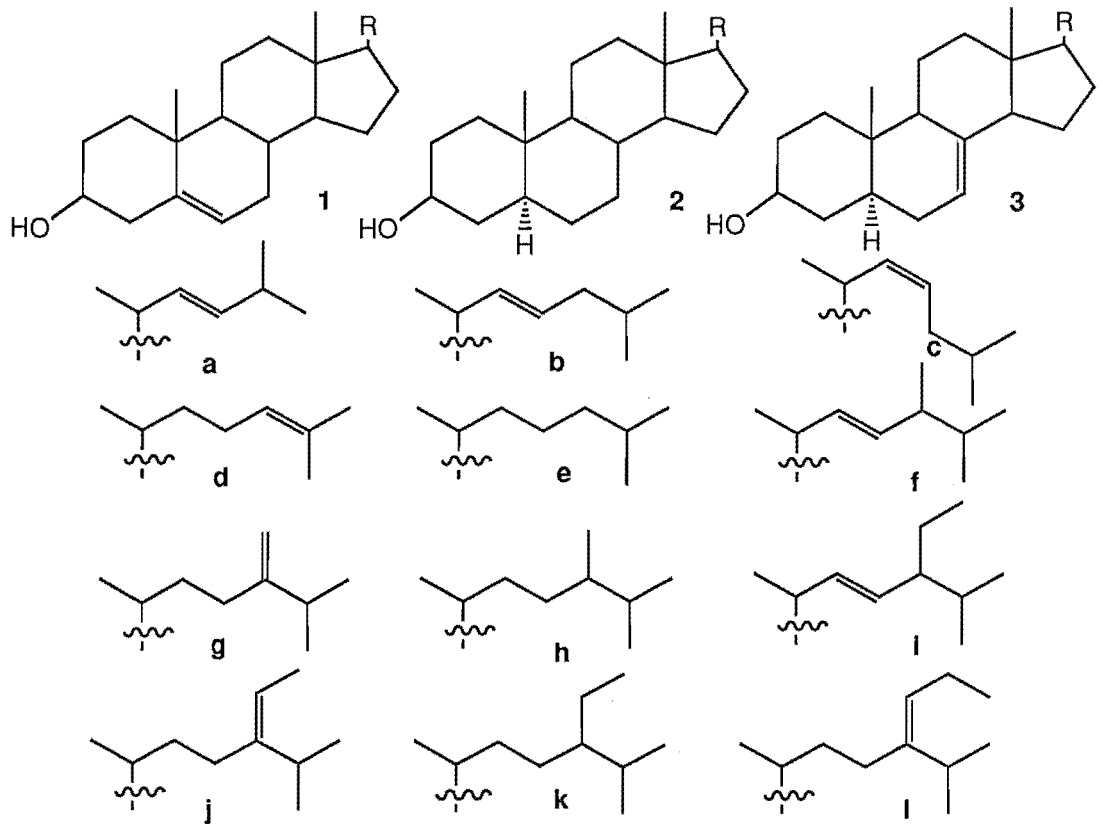
TLC SURVEY OF HYMEDESMIIDAE

All other samples in the Canterbury Marine Chemistry group's collection of the family Hymedesmiidae were screened by tlc and ^1H nmr spectroscopy to check for the presence of sulphate (**164**). The samples surveyed were *Stylopus* sp. 1, *Stylopus australis*, *Stylopus* sp., *Hymedesmia* sp. 1, *Hymedesmia* sp. 2 and three other samples identified as *Hymedesmia* sp. Sulphate (**164**) was found to be present in *Stylopus* sp. 1 in addition to *Stylopus australis*, but not in any *Hymedesmia* species, the only other genus in the family Hymedesmiidae, indicating perhaps that this compound is genus specific.

STEROLS.

Overall, the sterol sulphate (**164**) was found to be present in the sponge at a level comparable to that of the co-occurring sterols. It was desirable to investigate the sterol composition of *Stylopus australis* to see if other pregnane sterols were present and to give an indication as to possible precursors to (**164**). A comparison of the sterol composition with that of sponges which did not contain sulphate (**164**), was also considered useful. The sterol mixtures of *Stylopus australis, Hymedesmia* sp. 1 and *Hymedesmia* sp. 2 were examined for this purpose.

Sterol mixtures were isolated by silica gel column chromatography of chloroform extracts of the sponges. Analysis of the sterol mixtures was performed by gas-chromatography-mass spectrometry (gcms) of the trimethylsilyl (TMS) ethers of the sterols of each sponge.



(96) 1a (100) 1f (103) 1i (106) 1k (178) 3f
(97) 1b (101) 1e (104) 2e (176) 1c (179) 1l
(99) 1g (102) 1j (105)1h (177) 1d

The range of sterols encountered in this study and their gcms properties are summarised in Table 5.3. Three different sterol nuclei and twelve different sidechains were observed. The sterols are listed in order of increasing retention time on the gc capillary column. An analysis of the relative retention times and the mass spectral fragmentation patterns of each sterol enabled the assignment of molecular size, ring structure and position of double bonds[166,167]. For example, for sterols (**97**) and (**176**), the $M^{+\cdot}$ ion at 456 indicated that the parent sterol had a molecular formula of $C_{27}H_{44}O$. The base peak at m/e 111 was typical of a C_8H_{15} sidechain with a Δ^{22} double bond[167]. Prominent ions at m/e 129 and $[M-129]^+$ are characteristic of a Δ^5-3β-trimethylsilyloxysteroid and arise from the fragmentations shown in Figure 5.3[168]. The two sterols were differentiated by comparison of the relative retention times with literature values[166,167]. Side-chain epimeric sterols exhibit almost identical gcms data, so the relative configuration of the asymmetric centres of the side chain could not be determined[166].

Figure 5.3: Typical mass spectral fragmentations of a Δ^5-3β-trimethylsilyloxysteroid.

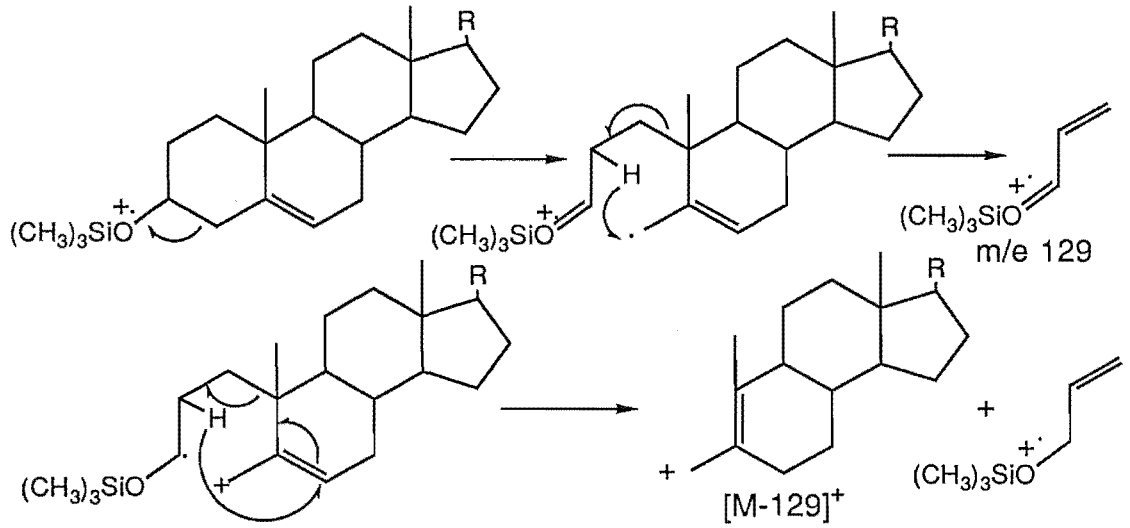


Table 5.3: GCMS Data of Sterol TMS Ethers

Sterol Structure	$M^{+\cdot}$	Relative Retention Time (cholesterol =1.000)	Typical MS Fragments[166,167] of TMS ethers.
(96)	442	0.870	313, 255, 129, 97.
(176)	456	0.960	351, 255, 327, 129, 111.
(97)	456	0.970	351, 255, 327, 129, 111.
(101)	458	1.000	353, 329, 247, 129.
(104)	460	1.003	305, 230, 215.
(177)	456	1.020	343, 327, 253, 213, 129.
(100)	470	1.030	341, 255, 129, 125.
(178)	470	1.060	343, 255, 129, 125.
(99)	470	1.068	386, 343, 341, 296, 257, 129.
(105)	472	1.071	367, 343, 261, 129.
(103)	484	1.090	484, 355, 351, 255, 139, 129.
(106)	486	1.130	381, 357, 275, 129.
(102)	484	1.140	386, 355, 296, 257, 129.
(179)	498	1.190	386, 296, 369, 129.

Table 5.4 summarises the sterols present in each species, listing them as percentage composition.

The same sterols are present in each of the two *Hymedesmia* species, but there are differences in sterol composition between *Stylopus australis* and the two *Hymedesmia* species.

Table 5.4: The sterol composition of Hymedesmiidae sponges.

	Percentage Composition In Each Species		
Sterols Present	*Stylopus australis*	*Hymedesmia* sp. 1	*Hymedesmia* sp. 2
(96)	2	5	6
(176)	6	2	3
(97)	10	7	11
(101)	24	13	15
(104)	+		
(177)		13	6
(100)	23	20	19
(178)	2		
(99)	12	35	35
(105)	3		
(103)	3	1	+
(106)	12	3	2
(102)	2	3	3
(179)	1		

+ indicates a trace amount of a particular sterol, <1%.

Stylopus australis contains 13 sterols of which most contain nucleus **1**. As for many sponges, the major sterol is cholesterol (**101**) with 24-methylene cholesterol (**99**) being the next most abundant. Minor quantities of both the completely saturated sterol, cholestanol (**104**), and a Δ^7 sterol (**178**) were present.

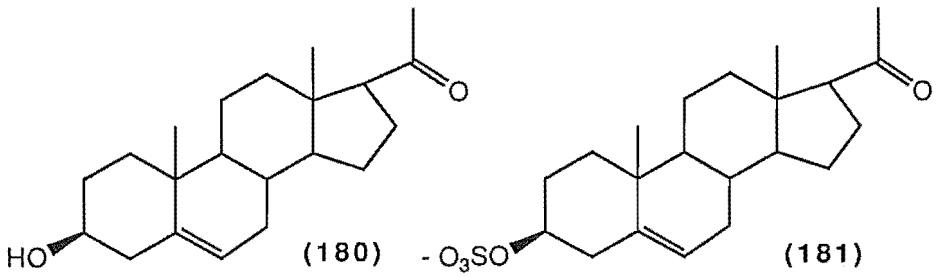
The sterol compositions of both the *Hymedesmia* species are very similar, with each possessing the same ten sterols. Relative quantities of each sterol differ between the two species but only for minor sterols. For each, 24-methylene cholesterol is the major sterol, followed by (**100**). Sterols in the two *Hymedesmia*

species are exclusively Δ^5. Both contain relatively large quantities of desmosterol (**177**), which is not present in *Stylopus australis*.

There is however, no major difference in the sterol composition of *S. australis* and the two *Hymedesmia* species which would give an indication as to precursors of (**164**) as hoped. It was thought that *S. australis* could contain other sterols with a pregnane sidechain which might be precursors to (**164**), but since this is not the case and as (**164**) is a major component of the sponge, it would seem that all such sterols are converted to (**164**). In light of the lack of biological activity of (**164**), it seems unlikely that it is an anti-fouling agent as postulated for compounds of this type in other sponge species[154].

ACTIVITY OF (**164**) AS NEUROSTEROID.

γ-Aminobutyric acid (GABA) inhibits nerve transmission in the mammalian brain[169]. Pregnenolone (**180**) and its sulphate ester (**181**) are thought to be synthesised locally in the mammalian brain where they have been characterised and are known to modulate contractions mediated by $GABA_A$ receptors in the isolated guinea-pig ileum[170]. As sulphate (**164**) only differs from pregnenolone sulphate in the presence of a 4β-hydroxyl group, a sample of (**164**) was sent to Professor Graham Johnston at the Department of Pharmacology, University of Sydney, Australia for testing in the isolated guinea-pig ileum assay.



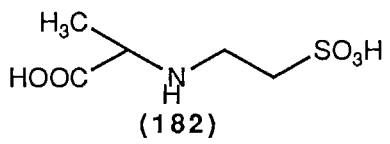
(**164**) was considerably less potent than pregnenolone sulphate in enhancing the action of GABA. Sulphate (**164**) enhanced GABA-induced contractions of the guinea-pig isolated ileum by 10% at both 10^{-7} and 10^{-8} M. Under the same conditions, pregnenolone sulphate has been found to enhance these contractions by 100% at 10^{-7} and 90% at 10^{-8} M[170]. Thus the presence of a 4β-hydroxyl group significantly reduces the activity of pregnenolone sulphate as a neurosteroid.

5.2 STUDIES OF THE SPONGE, *HYMEDESMIA* SPECIES 1

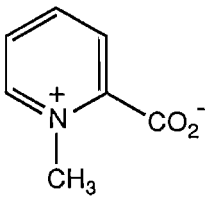
INTRODUCTION

There are only two genera in the sponge taxonomic family Hymedesmiidae: *Stylopus* and *Hymedesmia*. An investigation of an extract of the sponge *Hymedesmia* sp. 1 was undertaken for much the same reasons as outlined for *Stylopus australis* : the extract showed biological activity, and since no work on this genus had been reported in the literature, it was a potential source of new metabolites. In addition, any similarities in chemical composition between the genera *Stylopus* and *Hymedesmia* could be examined.

The investigation led to the isolation of N-(1-carboxyethyl)taurine (D-rhodoic acid) (**182**), as the major water soluble component of the extract. The ubiquitous marine metabolite homarine (**94**) was also isolated from the water soluble fraction of the extract, along with the amino acid taurine.



(182)



(94)

The biological activity of the extract was investigated, but pursuit of this was abandoned as the activity could not be well localised, despite the variety of chromatographic techniques attempted.

The sterol composition of the extract was examined for comparative purposes, and the results of this study are outlined in Section 5.1.

ISOLATION OF D-RHODOIC ACID (**182**)

The aqueous phase of a chloroform/water partition of a crude extract of *Hymedesmia* sp. 1 was further partitioned by reverse phase flash column chromatography. A ^1H nmr spectrum of the first fraction from this column showed the presence of several major components .

Gel permeation chromatography followed by reverse phase hplc, led to the isolation of (**182**) as the major water soluble component of the extract. (**182**) gave a red-pink spot when treated with ninhydrin spray. The structure of (**182**) was determined as follows. The ^1H nmr spectrum of (**182**) in D_2O (Figure 5.4) was very simple. A three proton doublet at 1.48-1.51 ppm was coupled to a one proton quartet

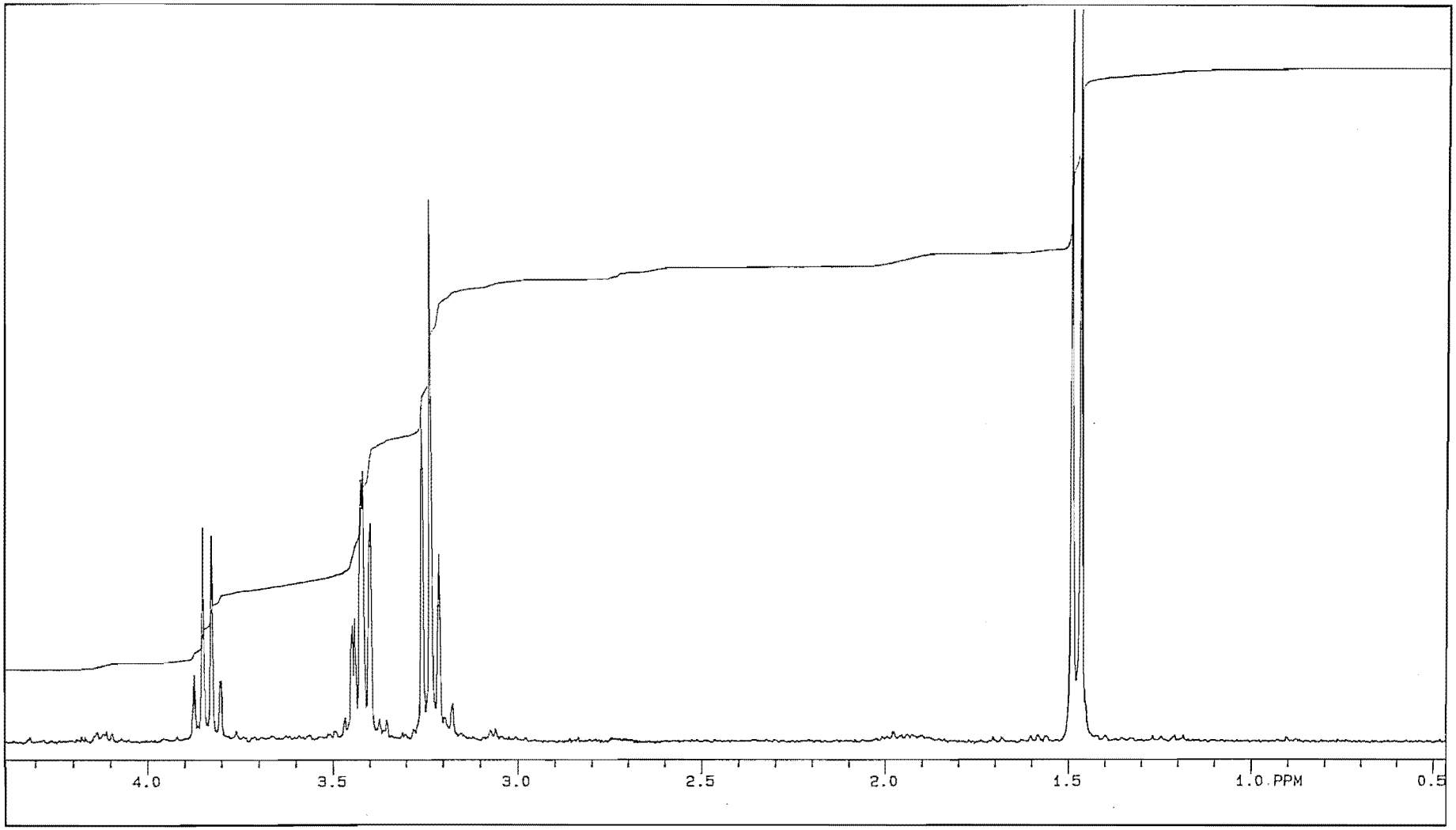


Figure 5.4: The ^{1}H nmr spectrum of compound (**182**) in D_2O.

(7.1 Hz), which depending on pH, was centred between 3.84-4.00 ppm. The only other signals in the ^{1}H nmr spectrum were two methylene triplets at 3.23 and 3.42 ppm (6.8 Hz). The ^{13}C nmr spectrum in D_2O showed a quaternary signal at 173.16 ppm, a tertiary signal at 57.25 ppm, two secondary signals at 47.40 and 42.34 ppm and a primary signal at 14.82 ppm. A HETCOR nmr experiment was used to determine that the methylene triplet at 3.23 ppm was attached to the carbon resonating at 47.40 ppm and that at 3.42 ppm, attached to the carbon signal at 42.34 ppm. Results of an XCORFE nmr experiment showed that the methine group resonating at 3.78 ppm was long range coupled to the secondary carbon at 42.34 ppm. This led to the proposal of the structural fragments shown in Figure 5.5.

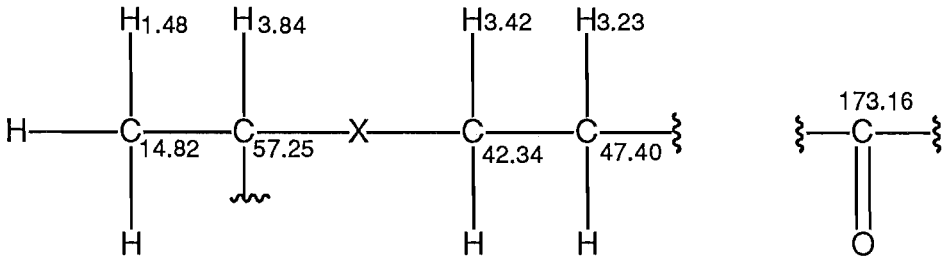


Figure 5.5: Structural fragments of rhodoic acid (**182**).

The chemical shift of the methine proton suggested that it was attached to a nitrogen atom. Comparison of the data with the ^{1}H and ^{13}C nmr data of alanine[171,172] and N-methyl taurine[173] inferred the stucture (**182**). The molecular formula of $C_5H_{10}NO_5S$ was confirmed by mass spectrometry. A negative ion **S**econdary **I**on **M**ass **S**pectrometry (SIMS) mass spectrum of (**182**) using a matrix of triethanolamine showed an $[M]^-$ of 196 and other ions at 234 $[M-3H+K]^-$ and 218 $[M-2H+Na]^-$.

The observation of strong bands in the ir spectrum of (**182**) at 1730 and 1200 cm^{-1} evidenced the presence of carbonyl and sulphinic acid functionalities respectively, while the observation of a negative ORD curve confirmed the chirality as D-, through comparison with that obtained previously[174].

Previous ^{1}H nmr spectroscopy of (**182**) isolated from abalone muscle, used the solvent trifluoroacetic acid (TFA). In this solvent, the methylene protons were not resolved into two well defined triplets, but appeared as a broad four proton singlet at 3.63 ppm[174].

Few reports of the isolation of rhodoic acid exist and its taxonomic distribution appears to be rather limited. The first report was in 1961 when it was

isolated by ion-exchange chromatography from three species of red algae, *Chondrus ocellatus*, *Neodelsea yendoana* and *Iridaea cornucopiae*. Permanganate oxidation of (**182**) yielded taurine, alanine and propionic acid[175]. Rhodoic acid, also known as tauropine, has since been found to have a very limited distribution among Rhodophyta and some species biosynthesise D-rhodoic acid, employing the enzyme rhodoic acid dehydrogenase to catalyze the reaction:

taurine + pyruvate + NADH $\longrightarrow$ D-rhodoic acid + NAD^+ + H_2O[176].

The abalone *Haliotis discus hannai* was also found to biosynthesise D-rhodoic acid by the same mechanism[177]. The enzyme responsible for the catalysis was isolated from muscle of ormers of the genus *Haliotis* by two groups of workers[178,179] and later from the pedicles of a brachiopod, *Glottidea pyramidata*. Taurine and pyruvate were shown to be the preferred substrates through kinetic studies[180].

Experiments involving anoxia in two different muscular tissues of *H. lamellosa*, showed that D-rhodoic acid was accumulated in the shell adductor muscle, used for lowering the shell for protection and righting the animal, while D-lactate was the glycolysis product in the foot muscle[181]. Thus D-rhodoic acid was the product of anaerobic metabolism in a specific muscle of the mollusk.

The isolation of D-rhodoic acid from *Hymedesmia* sp. 1 would appear to be the first report of this compound from a sponge. Whether it is a product of anaerobic metabolism in the sponge is not known, but it was the major water soluble component of the extract. A ^1H nmr spectrum of the water soluble fraction of an extract of *Hymedesmia* sp. 2, clearly showed the presence of this component also.

Rhodoic acid was inactive in the antiviral/cytotoxicity, P388 and antimicrobial assay systems.

ISOLATION OF HOMARINE

Fractions from the gel permeation chromatography column on the *Hymedesmia* sp. 1 water soluble partition, which eluted later than those containing rhodoic acid (**182**), contained mainly salt and one other major component. This component was identified as homarine (**94**) from its ^{13}C nmr spectra in D_2O[78]. Although homarine is a common marine metabolite found in many organisms, including bryozoans[79,80], tunicates[182], and nudibranchs[183], its isolation from *Axinella* sp. 2 (see Chapter Four) and *Hymedesmia* sp. 1 appear to be the first reports of its isolation from sponges.

ISOLATION OF TAURINE (**141**)

Taurine was identified in one of the hplc fractions in the isolation of rhodoic acid, by comparison of the ^{1}H and ^{13}C nmr data with literature values[143,144]. Taurine also gave a pink spot on tlc with ninhydrin spray.

(141)

BIOLOGICAL ACTIVITY OF SPONGE

The crude extract of *Hymedesmia* sp. 1 showed cytotoxicity (type C4) in the antiviral/cytotoxicity assay. Various combinations of normal and reverse phase column chromatography and gel permeation chromatography were attempted on this material, but all resulted in the spread of activity throughout the column fractions. A ^{1}H nmr spectrum of one of these active fractions in CD_3OD, was most uninformative, containing many broad unresolved signals.

An extract of *Hymedesmia* species 1 was screened by the procedure outlined in Chapter Seven of this thesis, and the results of this work are also outlined in Chapter Seven, p. 145.

As no suitable means of concentrating the biological activity could be found, and as no structural clues could be obtained from ^{1}H nmr spectroscopy of various fractions, work on the biological activity of the sponge was discontinued.

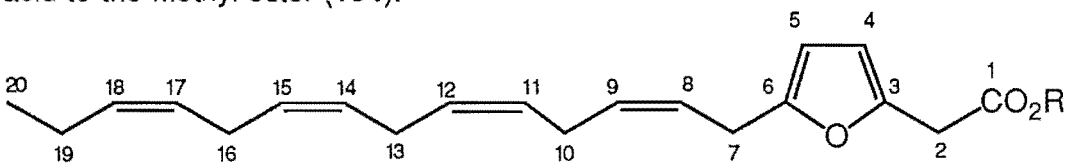
CHAPTER SIX

6.1 STUDIES OF THE SPONGE *HYMENIACIDON HAURAKI*

INTRODUCTION

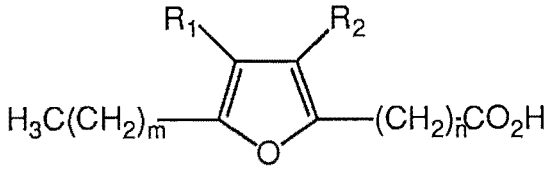
As part of the investigation of extracts from New Zealand marine invertebrates, an extract from the sponge, *Hymeniacidon hauraki* was examined. A literature search revealed that terpenoid isocyanides, some of which showed antimicrobial activity, had previously been reported from this genus[184]. Furthermore, as a number of novel sesquiterpenes exhibiting cytotoxic properties had been isolated from a sponge assigned to the genus *Hymeniacidon*[79], there was the definite possibility that *H. hauraki* might contain similar compounds. However, the sesquiterpene-containing sponge has subsequently been renamed as a *Eurypon* species[64].

The investigation led to the isolation of the new compound, (8*Z*, 11*Z*, 14*Z*, 17*Z*)-3,6-epoxyeicos-3,5,8,11,14,17-hexenoic acid (**183**), a furan fatty acid, which was responsible for the biological activity of the extract. The instability of the compound prevented full characterisation being achieved at first. However full characterisation was eventually achieved by conversion of a sub-sample of the fatty acid to the methyl ester (**184**).



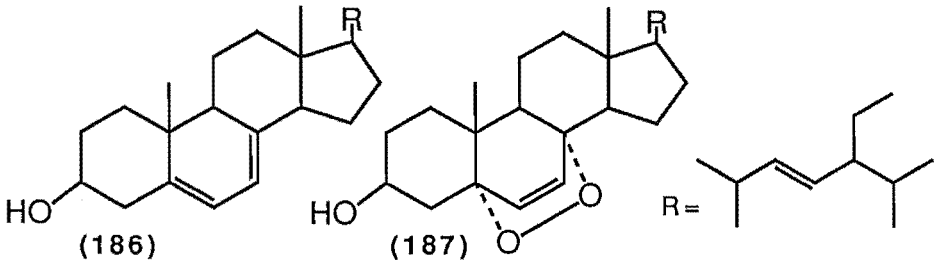
(183) R = H
(184) R = CH_3

Saturated furan fatty acids of the general type (**185**) have been isolated previously from several marine organisms including soft corals[185], ascidians[186] and scallops[186], but have been most commonly isolated from fish lipids[186,187]. The unsaturated furanoid fatty acid isolated from *H. hauraki* presumably arises through oxidation of the corresponding straight chain C20 fatty acid.



(185) R1, R2 = H, CH_3,
m = 2-5, n = 7-12

In the course of the investigation, the $\Delta^{5,7}$ sterol, (22E)-stigmasta-5,7,22-trien-3β-ol (corbisterol) (**186**) was isolated, along with its peroxide, 5α,8α-epidioxy-24ξ-methylcholesta-6,22-dien-3β-ol (**187**). This led to an examination of infra-specific sterol variation in *H. hauraki*, as the reported sterol composition of the species was quite different from that found in this work.



ISOLATION OF FURAN FATTY ACID (**183**)

Frozen *H. hauraki* sponge was extracted by blending with methanol/toluene and the resulting extract partitioned between chloroform and water. The organic phase was subjected to normal phase column chromatography on silica gel to yield a fraction, the tlc and ^{1}H nmr spectrum in $CDCl_3$ of which, revealed the presence of one major component. Further nmr spectroscopy, involving ^{13}C nmr, COSY, DEPT, HETCOR and decoupling experiments, led to the proposal of a compound with a disubstituted furan ring, (**183**).

The structure was proposed on the following evidence. The ^{1}H nmr spectrum of (**183**) (Figure 6.1) exhibited two mutually coupled one proton doublets at 5.93 and 6.13 ppm (3.2 Hz). These chemical shifts and coupling constant were appropriate for the protons on a furan ring, disubstituted as in compound (**183**)[188] and each of these signals showed a HETCOR correlation to carbon signals at 105.99 and 108.99 ppm respectively. The H4 doublet showed a COSY correlation to a broadened two proton singlet at 3.66 ppm (Figure 6.2) which in turn was correlated by a HETCOR experiment to a carbon signal at 33.80 ppm. That these signals arose from a methylene group was confirmed from the results of a DEPT nmr experiment, and the singlet in the ^{1}H nmr spectrum was thus assigned as arising from the H2 protons.

The H5 signal was further split into a doublet of doublets and showed a COSY correlation to a doublet at 3.39 ppm, assigned to the H7 protons. These protons exhibited a HETCOR correlation to a resonance at 26.28 ppm in the ^{13}C nmr spectrum and again a DEPT experiment verified the methylene grouping.

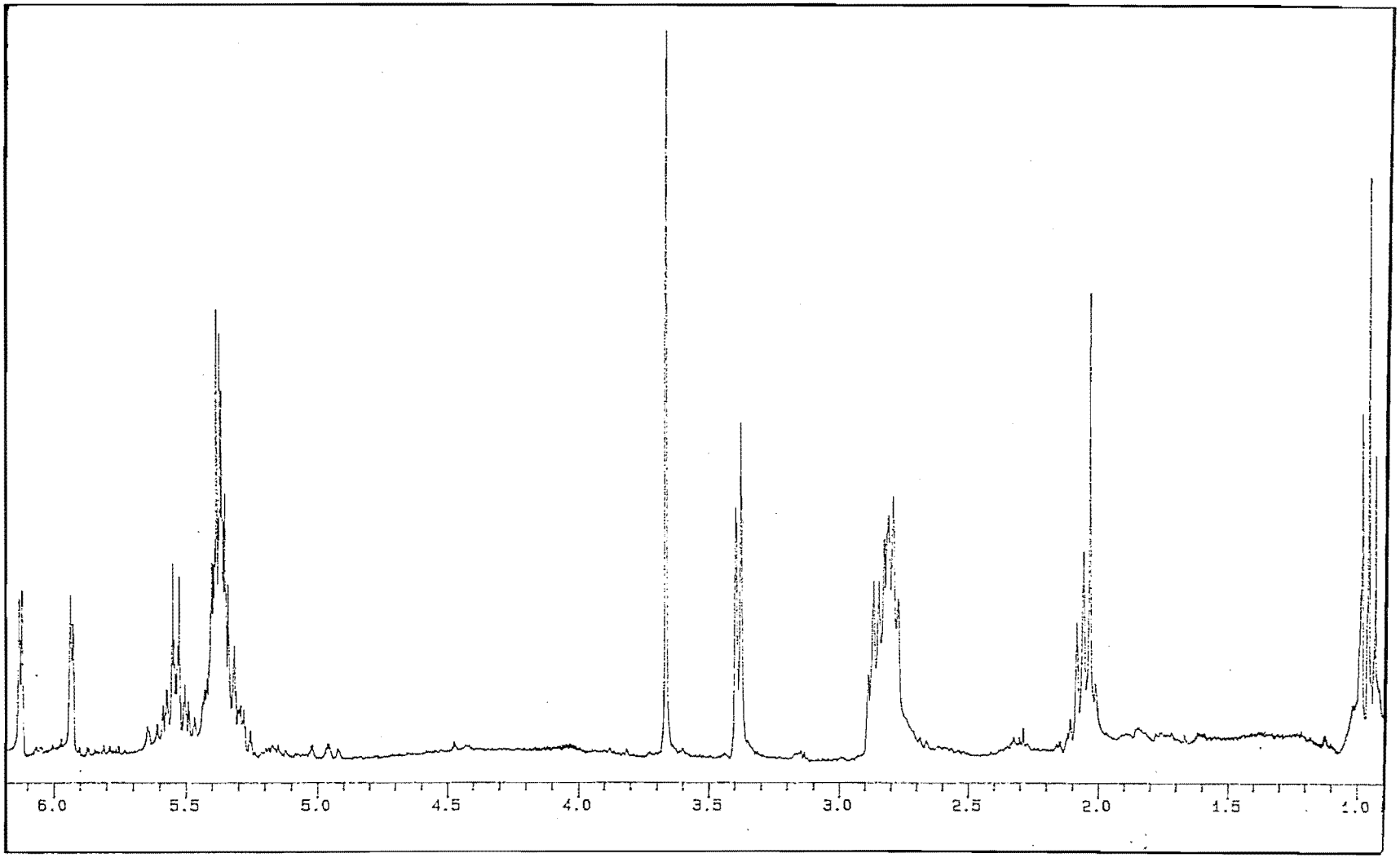


Figure 6.1: The ^{1}H nmr spectrum of compound (**183**) in $CDCl_3$.

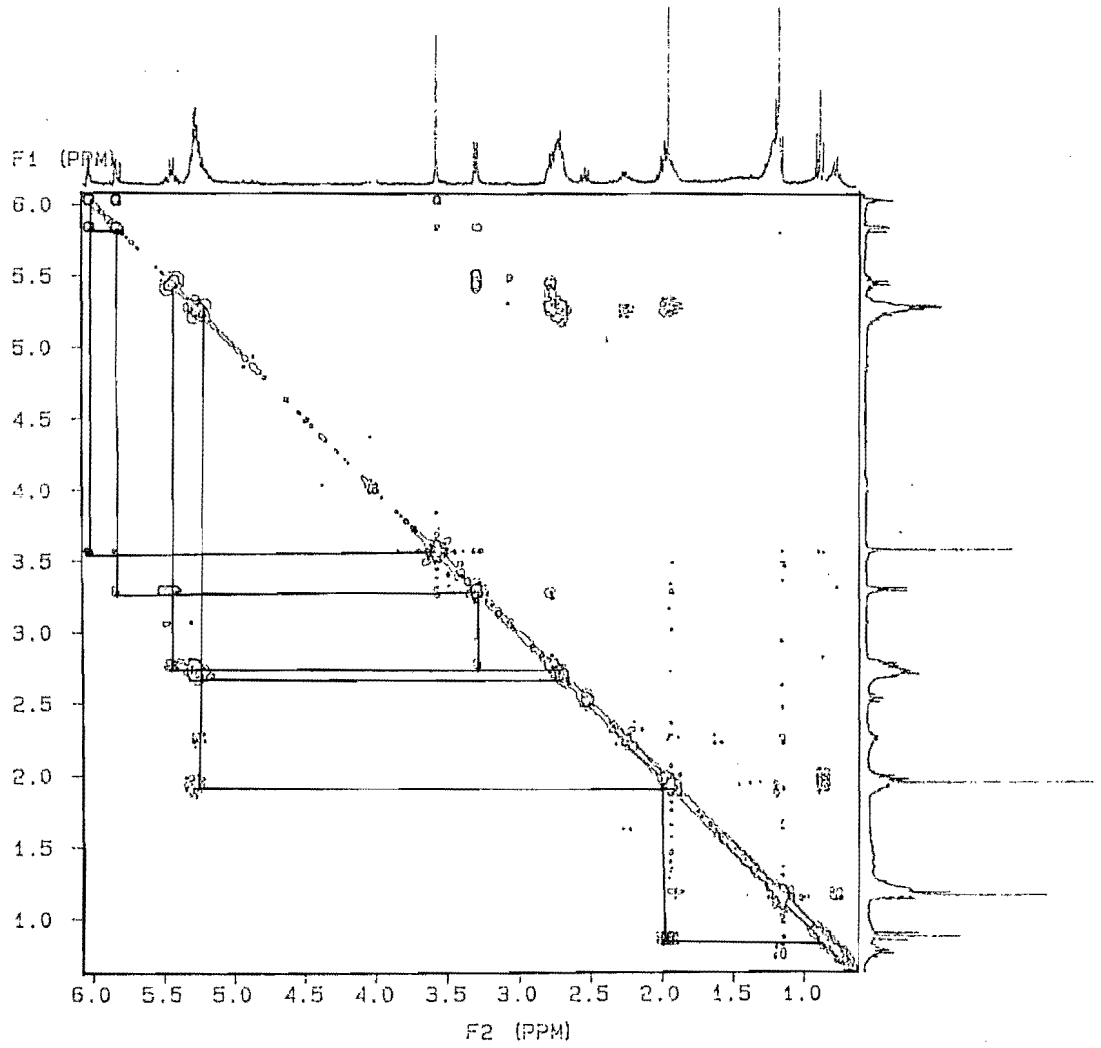
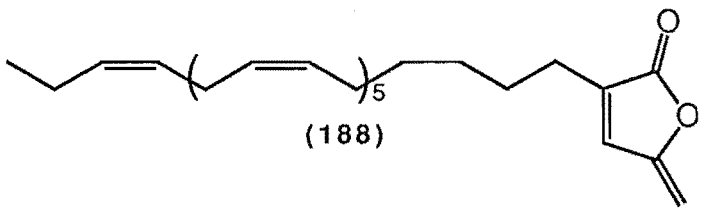


Figure 6.2: The COSY nmr spectrum of compound (**183**) in $CDCl_3$.

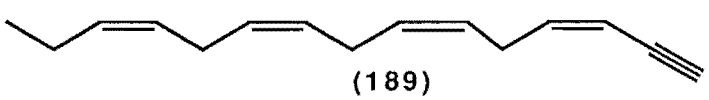
Results of a COSY experiment revealed that the H7 protons were coupled to a two proton multiplet at 5.54 ppm, one of which was attached to a carbon resonating at 124.64 ppm. The multiplet at 5.54 ppm, assigned to H8 and H9 was coupled to a complex multiplet at 2.82 ppm, again from the results of a COSY experiment. The integral of the multiplet indicated that six protons gave rise to the signal. This multiplet was coupled to another multiplet at 5.37 ppm which also integrated as six protons. Further correlation from this signal to a two proton multiplet at 2.06 ppm and a correlation from this multiplet to a triplet at 0.96 ppm, established the connectivity throughout the left hand side of the molecule.

The presence of a carboxylic acid functionality was suggested by the observation of a quaternary carbon signal in the ^{13}C nmr spectrum of (**183**) at 174.84 ppm and the observation of a strong band in the ir spectrum at 1710 cm^{-1}, in addition to a broad band at 3420 cm^{-1}. Other sharp bands in the ir spectrum at 990, 790 and 720 cm^{-1} were consistent with the strong δ C-H out of plane bending bands, characteristic of a furan[189]. Since the H2 protons were not coupled to any protons on an adjacent carbon, the carboxylic acid grouping must be attached to C2 and the carbonyl carbon was thus assigned as C1. Hence the structure of (**183**) was assembled. The ^{1}H and ^{13}C nmr spectral data for compound (**183**) are summarised in Table 6.1.

The stereochemistry of the double bond between C8 and C9 was determined to be *Z* from the magnitude of the coupling constant (7.3 Hz). The stereochemistry of the other double bonds in the chain could not be determined directly, due to lack of resolution in the ^{1}H nmr spectrum of (**183**). However, the ^{1}H nmr data of the alkyl chain of (**183**) fitted well with that of the alkyl chains of (6*Z*, 9*Z*, 12*Z*, 15*Z*, 18*Z*, 21*Z*)-2-tetracosa-6,9,12,15,18,21-hexaenylpenta-2,4-dien-4-olide (**188**)[190] and laurencenyne (**189**)[191].



(**188**)



(**189**)

Table 6.1: Nmr spectroscopic data for compound (**183**) in $CDCl_3$.

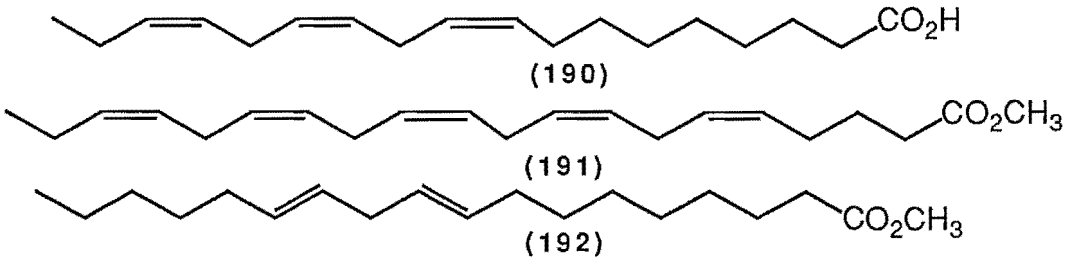
POSITION	δ ^1H	δ ^{13}C
1		174.84
2	3.66 s	33.80
3		154.12
4	6.13 d (3.2)	108.99
5	5.93 dd (3.2, 1.1)	105.99
6		155.66
7	3.39 d (6.3)	26.28
8	5.54 m (6.3, 7.3)	130.07
9	5.54 m	124.64
10	2.82 m	25.59
11	5.37 m	127.70*
12	5.37 m	127.78*
13	2.82 m	25.59
14	5.37 m	128.57*
15	5.37 m	128.47*
16	2.82 m	25.50
17	5.37 m	126.97
18	5.37 m	132.01
19	2.06 m (7.0, 7.6)	20.52
20	0.96 t (7.6)	14.14

() coupling constants in Hz.
*sequential values may be interchanged.

$^1J_{CH}$ correlations (HETCOR)

H2 <-> $C_{33.80}$
H4 <-> $C_{108.99}$
H5 <-> $C_{105.99}$
H7 <-> $C_{26.28}$
H8 <-> $C_{130.07}$
H9 <-> $C_{124.64}$
H10<-> $C_{25.59}$
H13
H16

Comparison of the ^{13}C nmr data of (**183**) with that of 9*Z*, 12*Z*, 15*Z*-octadecatrienoic acid (linolenic acid) (**190**)[192] and methyl 5*Z*, 8*Z*, 11*Z*, 14*Z*, 17*Z*-eicosapentaenoate (**191**)[192], facilitated the assignment of the double bond protons and confirmed the stereochemistry of each of them as *Z*-, as expected for a fatty acid derivative. The ^{13}C nmr resonance for the methylene groups between the double bonds was at 25.59 ppm, whereas if the double bonds were *E*- the methylene group resonances would be expected to be at lower field as in 9*E*, 12*E*-octadecadienoic acid methyl ester (**192**), where the signal for the methylene group between the double bonds resonates at 35.70 ppm[193].



(190) (191) (192)

Methylation of a sub-sample of (**183**) was attempted, using diazomethane in ether. Tlc analysis of the reaction mixture versus starting material revealed two distinct spots at low Rf in the starting material and a similar two spots, but at much higher Rf in the reaction mixture. Analysis of starting material and reaction mixture by nmr spectroscopy could not be performed immediately due to an instrumental malfunction, so both samples were dried under nitrogen, wrapped to exclude light and stored frozen until such time as further analyses were possible.

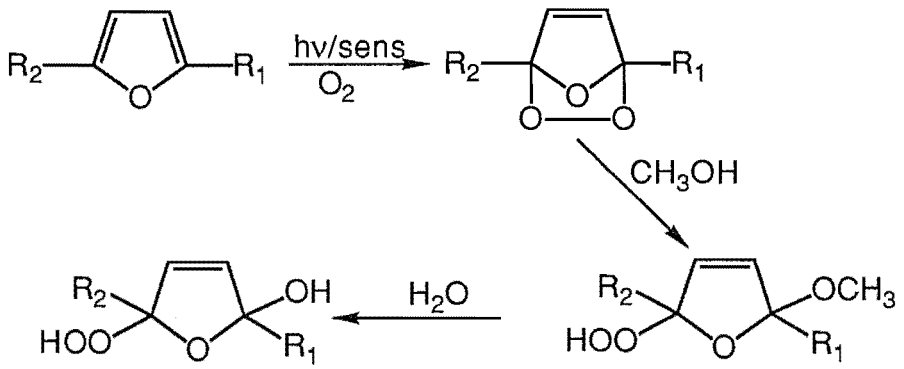
After a period of ten days, the ^{1}H nmr spectrum in $CDCl_3$ of each sample was obtained. In the spectrum of the starting material, all the signals were present, but the signal to noise ratio was much worse than that of the original spectrum obtained. For the methylated sample there was a new singlet at 3.73 ppm, indicating that methylation had occurred, but the signal to noise ratio was worse than that in the spectrum of the starting material, and the H4 and H5 resonances of the furan ring had virtually disappeared. The tlc evidence suggested that the compound had begun to undergo rearrangement or decomposition prior to methylation. It had been hoped to carry out nOe experiments to confirm the structural predictions made, but the entire sample decomposed before this could be achieved.

Prior to the attempted methylation of the nearly pure compound, (**183**) was shown to have moderate cytotoxicity (2+) at 20 µg per disc and weak cytotoxicity (±) at 5 µg per disc of type C3 in the antiviral/cytotoxicity assay.

RE-EXTRACTION OF SPONGE

The decomposition of furans in light and air in the presence of a sensitiser is well known and has been the subject of many studies[194-196]. Singlet oxygen reacts with the furan ring to form a peroxide, which can then undergo further reaction to form a variety of products depending on reaction conditions[197,198] (Scheme 6.1). It was therefore decided to attempt to reisolate sufficient of the furan compound to complete characterisation, by using an extraction procedure designed to exclude light and oxygen as much as possible.

Scheme 6.1: Reaction of 2,5-disubstituted furans with singlet oxygen[195,196].



A further sample of *H. hauraki* was extracted by blending with methanol/dichloromethane as previously, but the blender was flushed with nitrogen and an anti-oxidant, β-carotene was added to the extract prior to blending, in an attempt to halt decomposition of the furan. The extract was partitioned by reverse phase flash column chromatography and care was taken to exclude light from the fractions. Fractions shown to contain the furan compound by tlc and ^{1}H nmr analysis were recombined for further chromatography. As the compound could be lost through the carboxylic acid group causing irreversible absorption to silica gel, it was decided to use the less retentive DIOL phase instead. The fractions were therefore subjected to normal phase chromatography using DIOL as stationary phase. This led to the isolation of 1.5 mg of the desired compound, for further characterisation. Difficulty was encountered in obtaining a mass spectrum of the acid, so a small sample was converted to the methyl ester with diazomethane in ether. An EI mass spectrum of this material showed an $M^{+\cdot}$ ion at 328.2033, as appropriate for a molecular formula of $C_{21}H_{28}O_3$.

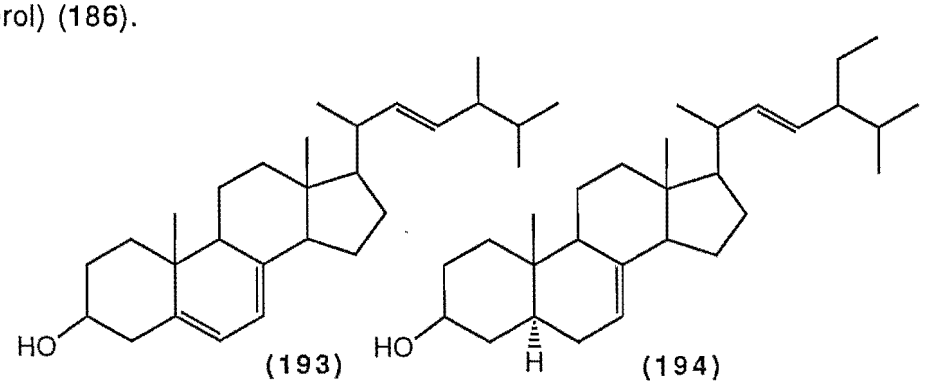
The pure compounds were submitted for assay in the antiviral/cytotoxicity and P388 assay systems. This time, the acid (**183**) exhibited the cytotoxicity type C9 in

the antiviral/cytotoxicity assay while the methyl ester (**184**) was inactive. In the P388 assay, compound (**183**) had an IC_{50} of approximately 13400 ng/ml, while the methyl ester again was inactive, implying that the carboxylic acid functionality is required for this compound to exhibit any cytotoxicity.

ISOLATION OF CORBISTEROL (**186**) AND ITS PEROXIDE (**187**)

Frozen *Hymeniacidon hauraki* sponge was extracted by blending with methanol/toluene, and the resulting extract further partitioned by reverse phase flash column chromatography. Tlc and ^1H nmr spectroscopy of two fractions from this column revealed sterols to be present and well-resolved peaks in the ^1H nmr spectra in the 2.0-2.5 and 5.0-5.4 ppm regions prompted further investigation. These two fractions were recombined and partitioned by column chromatography on silica gel. Information gained from ^{13}C, DEPT and COSY nmr spectra of one of the subsequent fractions was used to ascertain that a $\Delta^{5,7}$ sterol had been isolated and comparison of ^{13}C nmr data with literature values provided a good match for the sterol nucleus[197].

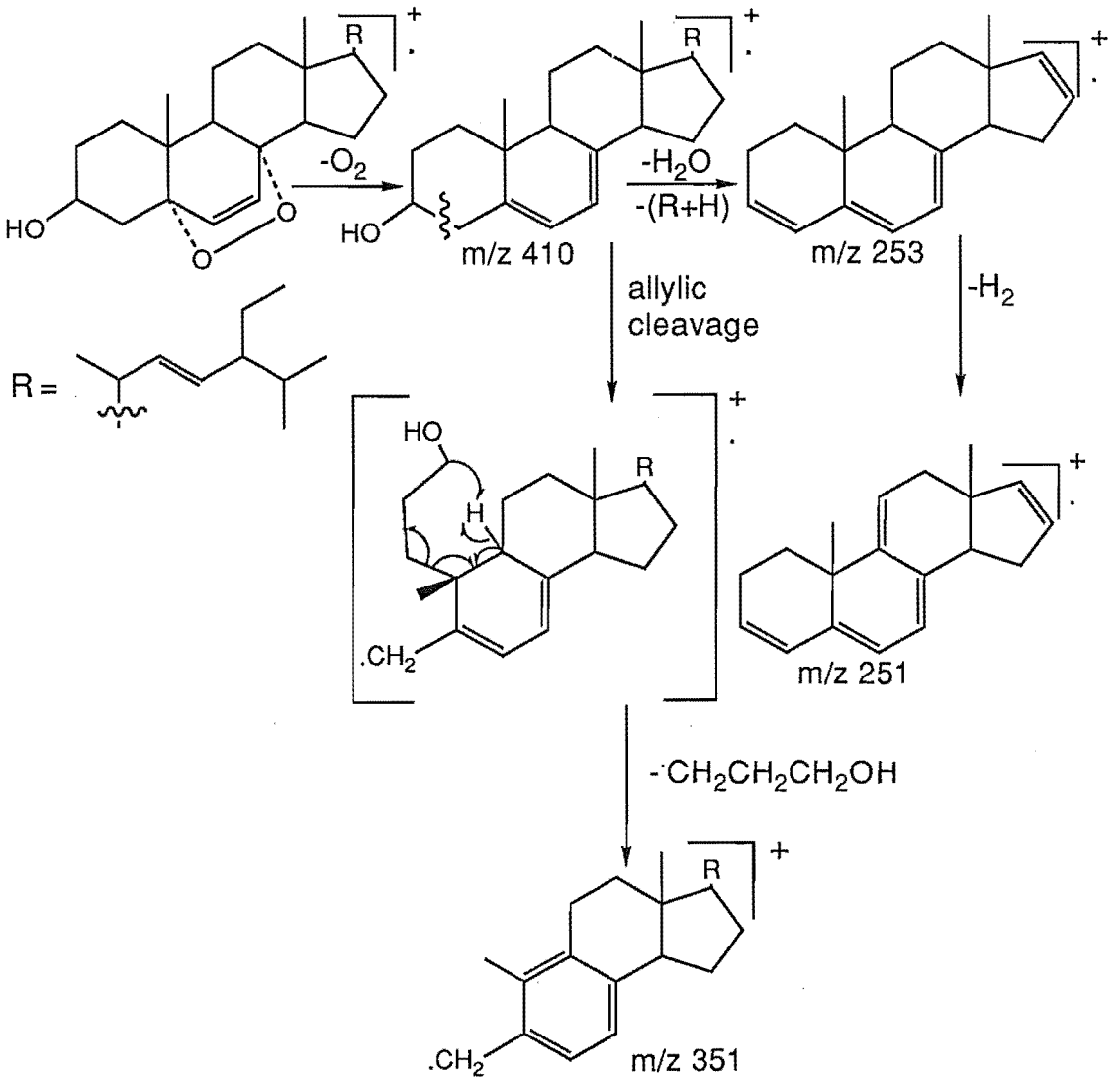
The ^1H nmr spectral data indicated that the sidechain contained a single double bond in a trans configuration, with a doublet of doublets at 5.10 ppm (J = 15.3, 8.2 Hz)[198]. That both olefinic carbons had one attached proton each was established from the results of a DEPT nmr experiment. Comparison of the data with those of the ergosterol (**193**) nucleus[197] and of the sidechain of chondrillasterol (**194**)[198,199] led to the structure being assigned as (22E)-stigmasta-5,7,22-trien-3β-ol (corbisterol) (**186**).



(193) (194)

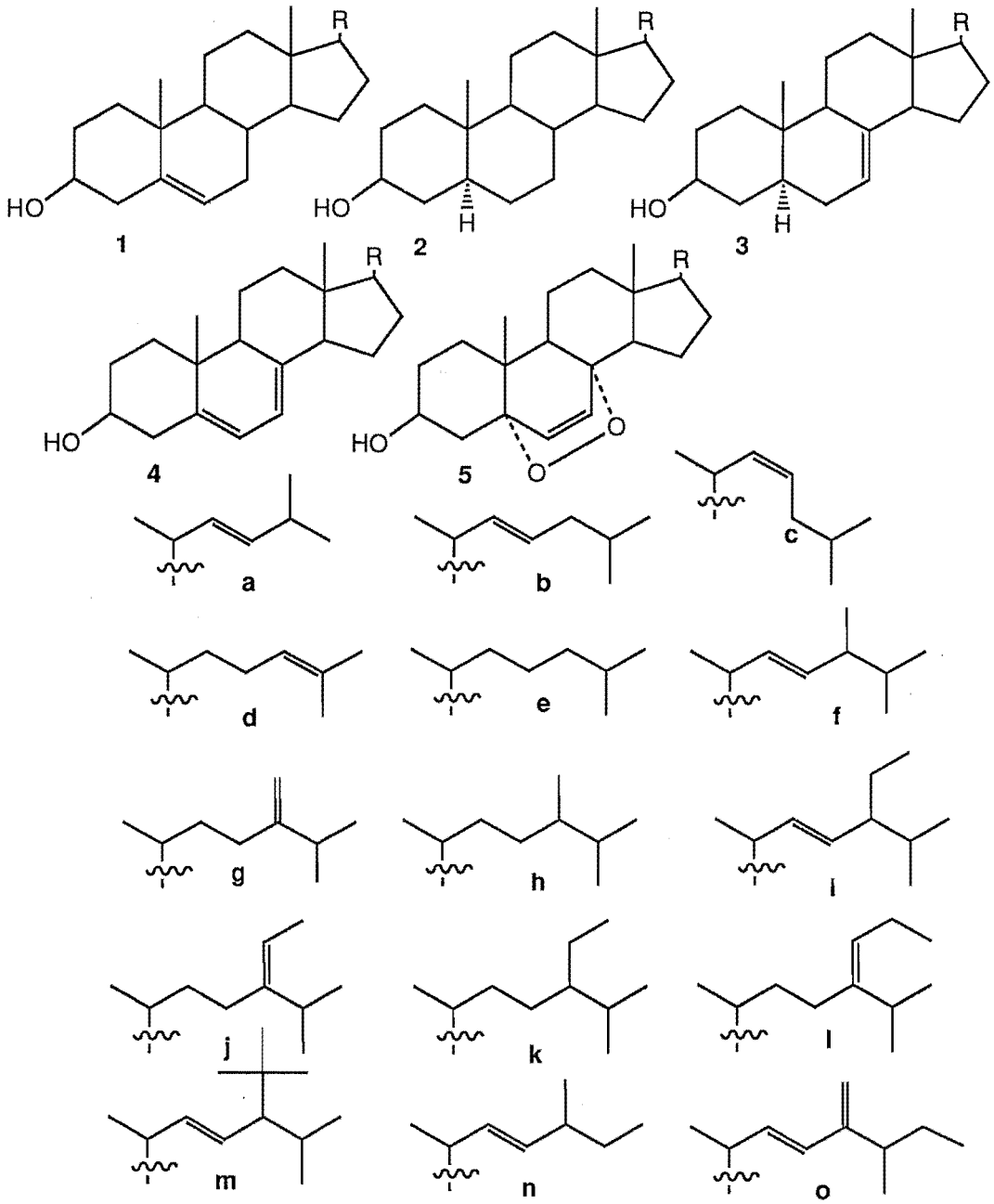
This assignment was confirmed by mass spectrometry. A high resolution CI mass spectrum of (**186**) using ammonia as reagent gas, showed an $[MH]^+$ ion at 411.3621, consistent with a molecular formula of $C_{29}H_{46}O$. A DEI mass spectrum showed an $[M]^+$ ion at 410 and fragment ions at 351 $[M\text{-}C_3H_7O]^+$ and 253 $[C_{19}H_{25}]^+$ (Scheme 6.2)[200].

Scheme 6.2: Some typical mass spectral fragmentations of $\Delta^{5,7}$ sterols and peroxides[200].



The ^{1}H nmr spectral data of another column fraction suggested that the peroxide of corbisterol, 5α,8α-epidioxy-24ξ-methylcholesta-6,22-dien-3β-ol (**187**) was present, with characteristic doublets at 6.23 and 6.50 ppm (J = 8.5 Hz) arising from the H6 and H7 protons of (**187**)[200].

Again mass spectrometry confirmed this. A DCI mass spectrum obtained using ammonia as reagent gas, showed an ion at 460 $[M+NH_4^+]^+$, while a DCI mass spectrum obtained using methane as reagent gas showed ions at 443 $[MH]^+$, 425 $[MH-H_2O]^+$, 411 $[MH-O_2]^+$, 377 $[M-H_2O-O_2-CH_3]^+$, 331 $[MH-C_8H_{16}]^+$ and 303 $[M-C_{10}H_{19}]^+$. A DEI mass spectrum showed an ion at 410, $[M]^+$ for (**186**), due to loss of oxygen from the molecular ion, probably by a retro-Diels Alder type fragmentation (Scheme 6.2)[200].



(96) 1a	(101) 1e	(106) 1k	(193) 4f	(197) 1n	(201) 2k
(97) 1b	(103) 1i	(176) 1c	(194) 3i	(198) 2b	(202) 3e
(99) 1g	(104) 2e	(177) 1d	(195) 1j	(199) 2g	(203) 3h
(100) 1f	(105) 1h	(186) 4i	(196) 1m	(200) 2j	

INFRA-SPECIFIC STEROL VARIATION IN *HYMENIACIDON HAURAKI*

The sterol composition of *Hymeniacidon hauraki* had been examined twice previously[166,201] and no $\Delta^{5,7}$ sterols were reported. The taxonomic identification of the sample was rechecked and confirmed as *H. hauraki*. This sample was collected from Takatu Point. Another extraction of the same sponge, but a different sample, this time collected from Leigh Reef, was therefore undertaken to check the sterol composition. The extract was partitioned between chloroform and water and then

further partitioned by normal phase column chromatography on silica gel. This extract again showed no signs of the $\Delta^{5,7}$ sterols isolated previously. At this stage, the taxonomic identification of the two samples was again examined by two different taxonomists, one of whom was Professor Bergquist, and it was verified that both samples were the same species, *H. hauraki.*

It was therefore desirable to analyse the sterol composition of each of the samples and to compare them with each other and with the previously reported data. Sterol mixtures were isolated by column chromatography on silica gel and the analyses were performed by gcms of the TMS ethers of the sterol mixtures of each sponge.

The range of sterols encountered and their gcms properties are summarised in Table 6.2.

Table 6.2: GCMS Data of *H. hauraki* Sterol TMS Ethers.

Sterol Structure	**$M^{+\cdot}$**	**Relative Retention Time (cholesterol =1.000)**	**Typical MS Fragments of TMS ethers[166]**
(96)	442	0.870	313, 255, 129, 97.
(176)	456	0.960	351, 255, 327, 129, 111.
(97)	456	0.970	351, 255, 327, 129, 111.
unidentified	454	0.990	
(101)	458	1.000	353, 329, 247, 129.
(104)	460	1.003	305, 230, 215.
(177)	456	1.020	343, 327, 253, 213, 129.
(100)	470	1.030	341, 255, 129, 125.
(193)	468	1.060	378, 363, 337, 253, 211, 131, 81, 74.
(99)	470	1.070	386, 343, 341, 296, 257, 129.
(105)	472	1.073	367, 343, 261, 129.
unidentified	482	1.090	
(103)	484	1.095	484, 355, 351, 255, 139, 129.
4?	484	1.110	
(186)	482	1.130	
(106)	486	1.140	381, 357, 275, 129.
(195)	484	1.150	386, 355, 296, 257, 129.

Table 6.3 summarises the sterols present in each sample and the percentage composition of each, as determined by gas chromatography. The same information reported for each of the two samples examined in the literature is also summarised in Table 6.3 for comparative purposes.

Table 6.3: Percentage Composition of the Sterol Mixtures of *H. hauraki* samples.

Sterols Present	Percentage composition of sterol mixture of *Hymeniacidon hauraki* samples			
	Leigh Reef	Takatu Point	Bergquist[166] *et al.*, 1980	Bergquist[201] *et al.*, 1986
(193)		10		
(186)		24		
(96)	+	+		
(97)	5	4	4	2
(176)	+	+		
(177)		4		
(101)	46	20	21	15
(100)	9	6	5	
(99)	4	8		14
(105)	4		8	9
(103)	4	6	9	2
(195)		3		
(106)	25	10	19	19
(196)				8
(197)				2
(198)				+
(104)	2	+	5	3
(199)				+
(200)				+
(201)			10	
(202)			+	
(203)			6	
(194)			10	20
+ 1?		+		
+ unidentified		+		
+ unidentified		+		
+ ($-C_{31}{}^{\circ}$)			+	
+ unidentified C_{28} and C_{31}		+		

+ denotes a trace amount, <1%.

As expected, the gcms data confirms the observation of markedly different sterol compositions of the two *H. hauraki* samples.

The Leigh Reef sample of *H. hauraki* contained ten different sterols and had a sterol composition similar to that reported for the species by Bergquist *et al*[166,201] with cholesterol (**101**) as the major sterol and (**106**) as the next most abundant. Bergquist reported a relatively high percentage of Δ^7 and ring-saturated sterols in her samples however, and in the sample studied no Δ^7 sterols were observed and the only saturated sterol present, (**104**) was a minor component of the mixture. This aside, the compositions are reasonably similar.

The sterol composition of the sample from Takatu Point which contained corbisterol (**186**) was quite different from that of the Leigh Reef sample and the compositions reported in the literature. Here there were sixteen different sterols and significant amounts of $\Delta^{5,7}$ sterols. Corbisterol was the major sterol of the sponge and ergosterol (**193**) was present as well. Other unidentified sterols present exhibited fragmentations in accord with those expected for $\Delta^{5,7}$ sterols[200]. Considerable quantities of cholesterol (**101**), 24-methylenecholesterol (**99**) and (**106**) were components of this sample as of the sample from Leigh Reef, and desmosterol (**177**) was present, in contrast to the Leigh Reef sample.

In the same study where the sterol composition of *H. hauraki* was first reported, Bergquist compares her findings of the sterol compositions of some sponges with the results of other workers on the same sponge species but from very different geographical locations, and finds "that sterol pattern is species-specific and that feeding regime does not influence the components of the pattern within a species"[166]. In other words, marked infra-specific sterol composition of sponges is not expected and has not been widely observed. In her later study however, Bergquist stated that although a re-examination of the sterol composition of *H. hauraki* confirmed the previous finding of mainly Δ^5 and Δ^7 sterols, the second sample "showed considerable variation in the levels of individual sterols present. Seasonal variation cannot be ruled out as a cause for this inconsistency"[201].

So although levels of any one sterol were somewhat variable, overall composition was relatively constant and no $\Delta^{5,7}$ sterols were observed. Environmental differences were not thought to be a significant factor in sterol composition and it is interesting to note that one of the samples studied by Bergquist was collected from an almost identical location to the sample containing $\Delta^{5,7}$ sterols. As corbisterol was the major sterol of the Takatu Point sample and not merely present

in a trace amount, contamination from other sponges is not a plausible explanation for its presence. It has been suggested that $\Delta^{5,7}$ sterols in sponges may be of symbiotic origin, as these sterols are known components of marine yeasts[166]. Verification of this would require examination of the sponge by electron microscopy, using special techniques to prepare the sample and this facility and the required expertise were not available.

It has also been demonstrated by a series of double labelling experiments that sponges are capable of converting Δ^5 sterols into $\Delta^{5,7}$ sterols. That this function is performed by the sponge itself and not by symbiotic algae or fungi was inferred from an examination of the sponge by electron microscopy[202]. Thus it is possible, since a "typical" *H. hauraki* sample contains predominantly Δ^5 sterols, that these are sometimes converted to $\Delta^{5,7}$ sterols by the sponge.

A number of samples of *H. hauraki* from a limited variety of geographical locations, consisting of several individuals per sample were available in the University of Canterbury Marine Chemistry group's collection. Each of these samples was separated into individuals by the group's taxonomist and the individuals were stored separately. A small scale chloroform extraction of each individual was performed and the ^1H and ^{13}C nmr spectra of the extract obtained to check for the presence of $\Delta^{5,7}$ sterols. In every case, signals characteristic of these sterols were noted in the nmr spectra of the extract (Figures 6.3 and 6.4) and in some of the extracts the presence of peroxides was noted also, confirming that the isolation of $\Delta^{5,7}$ sterols from the Takatu Point sample was not an exceptional case.

For a detailed study of the infra-specific sterol variation in *H. hauraki* in the future, a carefully designed sampling programme would need to be carried out. Individual sponges from varying geographical locations and depths would need to be collected during different seasons to cover as wide a range of environmental conditions as possible. Care would have to be taken that each sample was indeed an individual and that no contamination occurred before examining each sample by electron microscopy, extracting it and examining the sterol composition by gcms. Such a study was beyond the facilities and expertise available, so at the time of writing, has not been carried out.

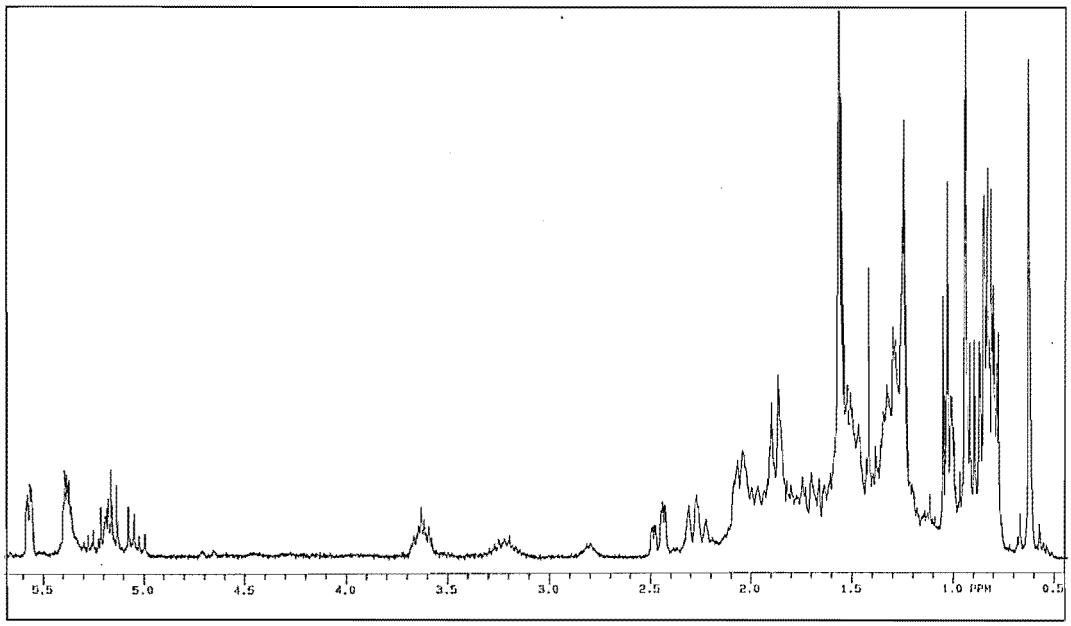


Figure 6.3: The ^{1}H nmr spectrum of corbisterol (**186**) in $CDCl_3$.

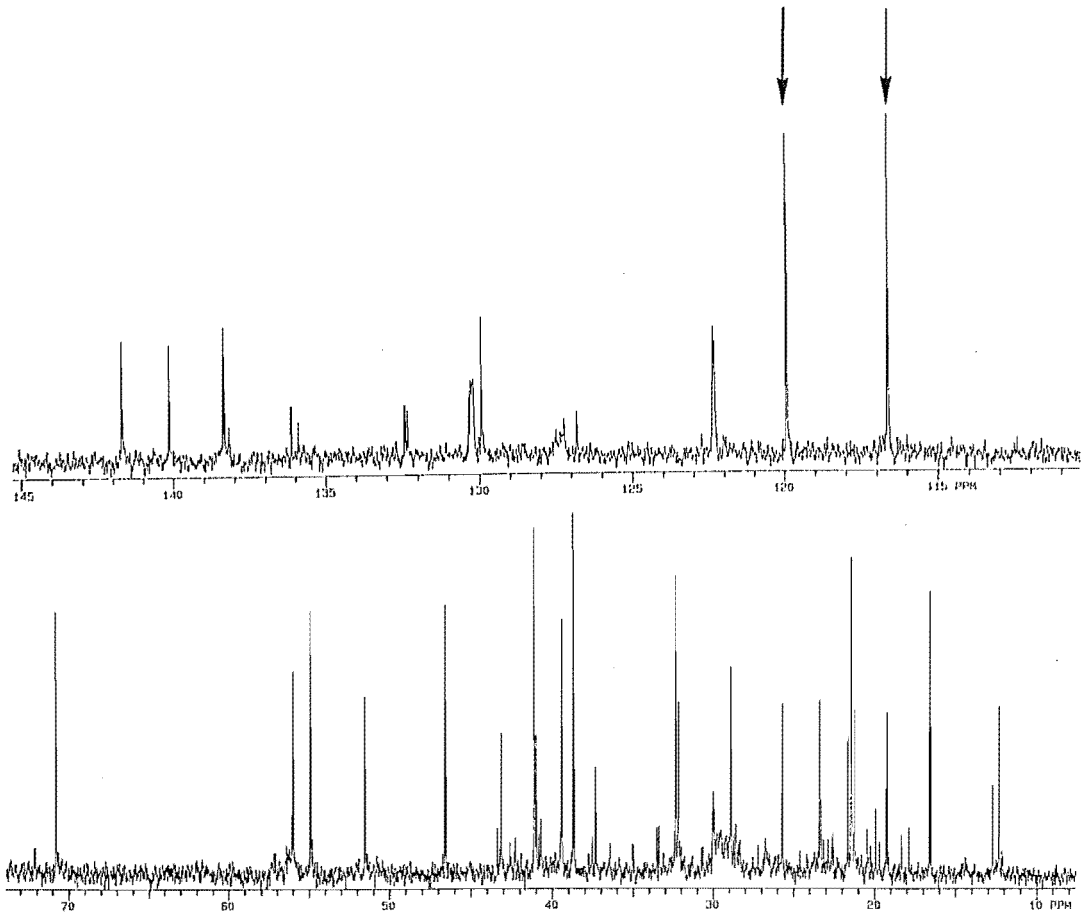


Figure 6.4: The ^{13}C nmr spectrum of corbisterol (**186**) in $CDCl_3$, with characteristic signals indicated.

ORIGIN OF STEROL PEROXIDES

A number of theories have been advanced to explain the isolation of sterol peroxides from sponges. Djerassi *et al.* found several sponges to contain almost exclusively sterol peroxides and concluded that these were actual sponge components[200]. Later however, Djerassi *et al.* found that a re-examination of freshly lyophilised samples of these sponges showed only the presence of $\Delta^{5,7}$ sterols and no peroxides were detected[202]. Therefore the isolation of sterol peroxides from sponges is most probably the result of oxidation of the conjugated diene system of sterols containing nucleus 4 during work-up or on storage. This is substantiated by the present study, in which $\Delta^{5,7}$ sterols only were detected in some samples investigated, and peroxides in addition to $\Delta^{5,7}$ sterols, detected in others.

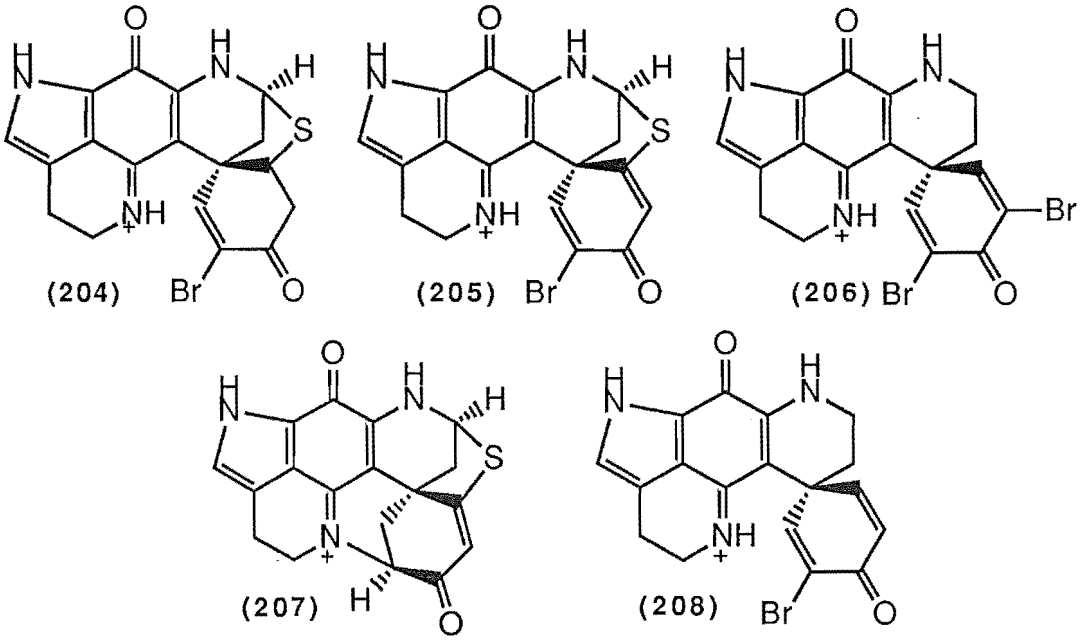
A recent study, in which sterol peroxides were isolated from the Cnidarian *Actinia equina*, concluded that *A. equina* transforms dietary sterols into peroxides on the basis of a carbon-14 incorporation experiment[203]. The sterols of the mussel *Mytilus edulis*, the chief prey of *A. equina* were examined and found to be exclusively Δ^5, but with an almost complete coincidence of sidechains with the peroxides isolated from *A. equina*. That these peroxides did not arise from the sterols of *A. equina* was concluded after an examination of the sterol composition of *A. equina* did not reveal the presence of most of the sidechains observed in the peroxides. 4-[^{14}C]-cholesterol was injected into *A. equina* specimens and the sterol and peroxide fractions later isolated. Most of the ^{14}C label was still present in the sterol fraction, but some radioactivity was detected in the peroxide fraction and the authors concluded that the *in vivo* transformation of sterols to peroxides was occurring. Contrary to the authors' statement, this *in vivo* transformation was not proven but only one possible explanation for the isolation of the sterol peroxides and not the most plausible. It is the opinion of this author that in light of the results of other workers[202], it is more likely that *A. equina* converts dietary Δ^5 sterols to $\Delta^{5,7}$ sterols and that the isolated peroxides arise through the oxidation of these sterols on work-up. Experiments involving injection of labelled $\Delta^{5,7}$ sterols into *A. equina* should either prove or disprove this assertion.

CHAPTER SEVEN

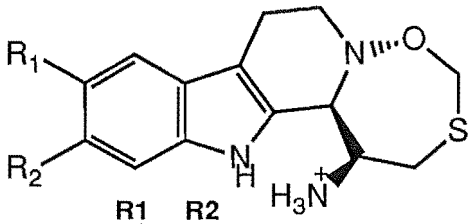
7.1 "FINGERPRINTING" OF MARINE NATURAL PRODUCT EXTRACTS

INTRODUCTION

During the course of searching for biologically active compounds from marine invertebrates, the Marine Chemistry Research group at the University of Canterbury has experienced problems that are common to all branches of natural products chemistry, *viz* the re-isolation of known compounds and therefore the duplication of effort between research groups. A case in point was the isolation of prianosin A (**204**) from the sponge *Prianos melanos* by Kobayashi and co-workers[204]. This



compound had previously been isolated by the Canterbury group as one of a series of biologically active compounds (**204-208**) from sponges of the genus *Latrunculia*[205-207]. Another example is the isolation of the eudistomins (**59-76**) by Rinehart *et al* from the ascidian *Eudistoma olivaceum*[57]. Some compounds of this series (**61**), (**63**) and (**209**) which were antiviral, were subsequently isolated by the Canterbury group from the ascidian, *Ritterella sigillinoides*[58]. This problem has been encountered by most natural products research groups at some stage, and many other such examples exist.



	R1	R2
(61)	OH	Br
(63)	H	Br
(209)	H	H

The isolation and identification of a biologically active compound from a marine organism is an exercise that involves considerable time and effort. More often than not, the compound is present at a low level in the extract of an organism, and therefore many purification steps are required to obtain a pure compound. If the compound responsible for the biological activity of the organism has been isolated previously, the effort involved in the isolation duplicates the previous input. Although the isolation of known biologically active compounds from organisms not previously known to contain that class of metabolites is of value from a chemotaxonomic or ecological perspective, the major aim of this and some other groups is to isolate and identify new biologically active marine metabolites which might have potential as future pharmaceutical agents. This problem of duplication of effort can potentially hinder progress in the fight against cancers and other diseases, and does nothing to enhance the morale of the hapless researcher, who may have spent hours of painstaking work on the isolation of a known metabolite. It is therefore desirable to identify known active components of an extract at as early a stage as possible, to minimise both effort and disappointment. We have coined the phrase "fingerprinting" to describe this overall process of dereplication of extracts. This overall process includes various phases, but undoubtably the most important is "chemical screening", the chemical equivalent of screening extracts for biological activity. This procedure, along with taxonomy, is critical in the dereplication process. A flow sheet, (Figure 7.1) describes the general approach our group takes towards the process of dereplication.

Taxonomy is the starting point in this approach, as closely related organisms often contain similar components. If the taxonomic classification of an organism is known, this can be used to search the literature for related organisms, to give some idea of the types of compounds which may have been found in earlier extracts of the organism under consideration. In some cases however, the same compound has been found in organisms that are unrelated taxonomically, and chemical components

may vary even within a species, depending on such factors as habitat (diet) and season. Obtaining reliable taxonomic identifications of organisms is no simple matter, as marine invertebrate taxonomy is a complex field and even experts in the area do not always agree. Taxonomic relationships are therefore not a totally reliable guide in this type of work and should be treated with caution.

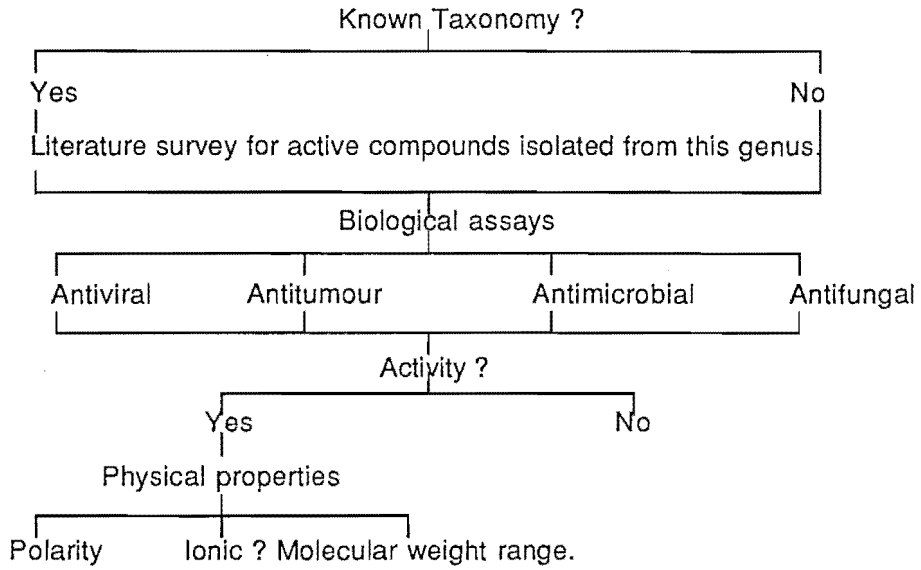


Figure 7.1: The general approach to the dereplication of extracts taken by the Canterbury Marine Chemistry group.

The question of which methods to employ in the isolation of the biologically active component of an extract presents further difficulties to the research worker. The biologically active component may be polar or non-polar, of high or low molecular weight, acidic or basic and the best method of approach to the isolation will vary with both the nature of the biologically active component and the nature of the other components of the extract in question. For example, if it is known that the compound responsible for the biological activity of the organism is non-polar, a good initial approach may be to partition the extract between water and a relatively non-polar solvent such as dichloromethane, thereby removing much mass quickly in the initial step of purification.

In an effort to address the problems of dereplication, a chemical screening procedure has been developed. The aims of the procedure are to aid in the prompt identification of known biologically active compounds, and to give information on the properties of unknown active components of an extract. Based primarily on chromatographic behaviour, the procedure utilises a variety of small scale chromatographic separations on the crude extract, monitored by bioassay, to

generate an activity/chromatography profile for a given extract. Valuable information is obtained on the chromatographic properties of the active component, thus defining the best approach to its isolation. This profile can then be compared with those known for active marine metabolites and if a match is found, the presence, (or absence) of the known compound can be checked quickly. The value of this is amplified greatly if taken in conjunction with the marine literature and taxonomic data. The three assay methods outlined in Chapter One of this thesis were used in this work.

THE SCREENING PROCEDURE

In the screening procedure, taxonomy was used initially to identify the organism, providing one means of differentiation between extracts. A small scale extraction was then performed and sub-samples of the crude extract assayed for biological activity in antimicrobial, antiviral/cytotoxicity and P388 assay systems to furnish another means of extract differentiation. Stability of the biological activity was checked by reassay of the crude extract one week later.

The extract was sub-divided for chromatography. Small scale column chromatography was carried out, using silica gel, C18 reverse phase and phenyl reverse phase material as stationary phases, and taking crude fractions of a standard volume and solvent composition. The crude extract was adsorbed on to a neutral solid support, celite, (diatomaceous earth), and dry loaded onto the columns. This approach facilitates handling of the extract, increases the surface area and avoids solubility problems or solvent effects. Fractions were assayed for activity and the results used to construct an activity/chromatography profile for each extract.

Extracts for which the active components were known were used as standards. They were selected to represent a wide range of polarities of the active components. Standard organisms were seven sponges: *Latrunculia brevis*[205], *Mycale* sp. I[208], *Strongylacidon* sp. I[209], *Ircinia novaezelandiae*[210], *Tedania diversirhaphidiophora*[211], *Axinella* sp. 2 (see Chapter Four of this thesis), *Petrosia hebes*[212], and an ascidian, *Ritterella sigillinoides*[58]. "Unknown" extracts were of two different types. The first type were extracts for which the identity of the extract and of the biologically active component were known, (but not by the experimentalist), while the second type were extracts which had been identified taxonomically, but the

source of the biological activity had not been determined. Extracts of this type studied were those of *Raspailia* sp. 5, *Stylopus australis*, and *Hymedesmia* sp. 1.

The results were collated as an activity/chromatography profile for each extract (Figures 7.2-7.13) as follows. For each crude extract, typical results in all three biological assay systems are shown. Antiviral/cytotoxicity results are expressed in the manner outlined in Chapter One of this thesis. Antimicrobial assay results are expressed in the form, *E. coli* score, *B. subtilis* score, *P. aeruginosa* score, *C. albicans* score and the weight of sample impregnated onto the disc in micrograms. Using the P388 assay data, the percentage activity in each fraction is calculated using the formula: % activity = a/b x c/d x 100

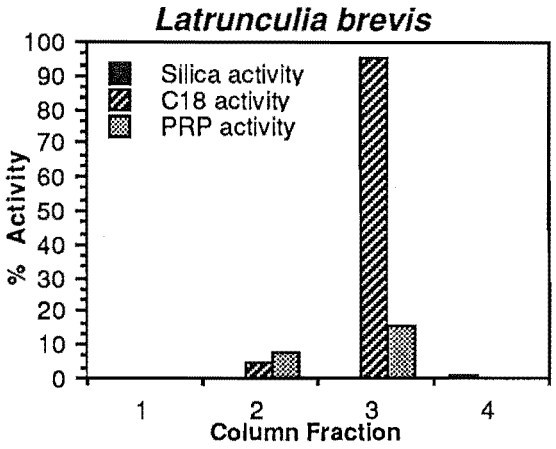
where
a = mass of column fraction (mg).
b = mass loaded onto column (mg).
c = P388 assay result of crude extract (ng/ml).
d = P388 assay result of column fraction (ng/ml).

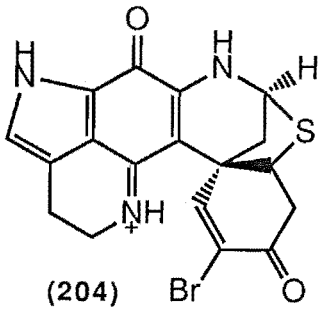
This method of expressing the results was adopted preferentially for the reasons of clarity of presentation, better quantitation of activity recovery and as it is the assay of choice in the Canterbury group for determining the potential of an extract for further work.

The graphs are therefore compiled using the P388 assay data, except in the cases of *Stylopus australis* and *Hymedesmia* sp. 1, where, as no P388 activity could be detected, the graph is compiled from the antiviral/cytotoxicity data in the following manner. Activity levels were expressed as a value representing the concentration of activity with respect to the crude extract. For example, if the minimum active dose of crude extract was 100 μg per disc, and the minimum active dose of a certain fraction was 20 μg per disc, the activity of this fraction was expressed as 5. This method shows the concentration of activity, and its loss in some cases, where the activity of a fraction was less than that of the same dose of the crude extract. For the *Stylopus australis* extract, the crude extract exhibited no activity, so the activity was standardised against the most active fraction.

DISCUSSION OF RESULTS

The activity profiles of the standard extracts (Figures 7.2-7.13) correspond well to the expected chromatographic behaviour of the appropriate active compounds. Each different active component was clearly distinguishable by its activity profile and the two standard extracts containing the same active component had very similar profiles.

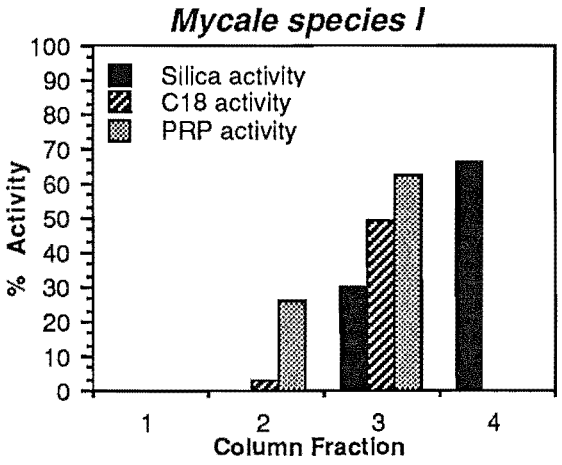




Antiviral/cytotoxicity assay:	?	?	ww	C8	200
	±	±	2+	C1	20
	-	-	-		2
P388 assay (IC_{50}) (ng/ml):		4371			
Antimicrobial assay:	-	*	-	-	200

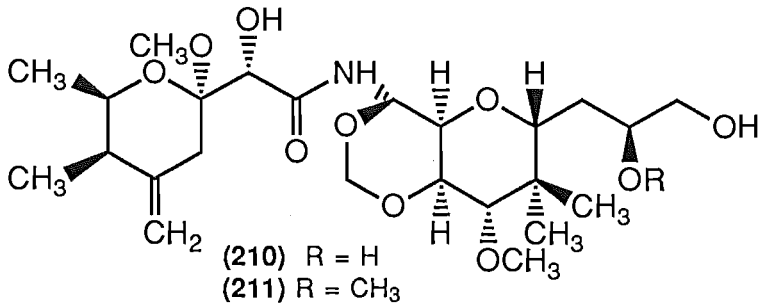
Figure 7.2: Activity/chromatography profile of *Latrunculia brevis*.

The compound responsible for the biological activity of *Latrunculia brevis* is (**204**), one of a series of compounds found in *Latrunculia* species, all of which are biologically active. This compound has medium polarity, as its chromatographic behaviour shows, with activity concentrated in fractions 2 and 3 of C18 reverse phase and phenyl reverse phase columns, and the recovered activity in fraction 4 of the silica column. Recovery of the activity from silica is poor, partly as solvents used are not polar enough to elute all of this compound and partly as the compound adheres to silica gel.



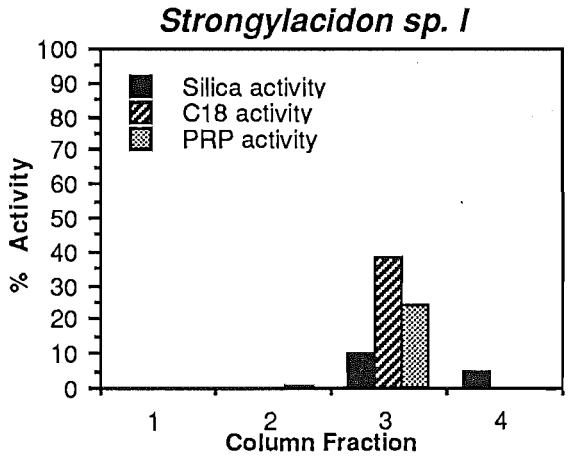
Antiviral/cytotoxicity assay:	ww	ww	±	C7*	2
	-	-	-		0.5
P388 assay (IC_{50}) (ng/ml):		160			
Antimicrobial assay:	-	-	-	-	200

Figure 7.3: Activity/chromatography profile of *Mycale* sp. I



(210) R = H
(211) R = CH_3

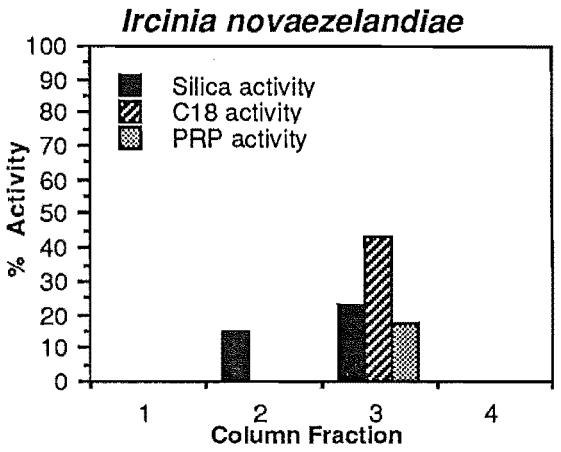
The biologically active compounds in *Mycale* species I are the mycalamides, (**210-211**), compounds of mid-range polarity, but slightly less polar than (**204**). The chromatographic behaviour reflects this, with activity concentrated mainly in fraction 3 of the C18 reverse phase column, fractions 2 and 3 of the phenyl reverse phase column and in fractions 3 and 4 of the silica column. These compounds are very potent with an IC_{50} of 0.5 ng/ml for (**210**) and 0.1 ng/ml for (**211**).



Antiviral/cytotoxicity assay:	3+	3+	±	C7*	200
	-	-	-		50
P388 assay (IC_{50}) (ng/ml):		7576			
Antimicrobial assay:	-	-	-	-	200

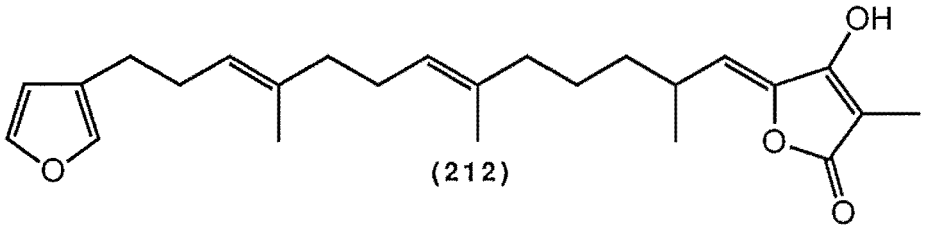
Figure 7.4: Activity/chromatography profile of *Strongylacidon* sp. I.

Strongylacidon species I contains the same active components as *Mycale* species I, but at a much lower level (~ 1%). The assay results for the crude extracts indicate this, with IC_{50} values of 7576 and 160 ng/ml respectively. The activity profiles for the two extracts however are very similar, as would be hoped if the screening procedure is to be of use. This is a good example of the same compounds occurring in species which are not particularly closely related taxonomically. Both *Mycale* and *Strongylacidon* species are of the order Poecilosclerida, but the genus *Mycale* is a member of the Mycalidae family, while *Strongylacidon* is from the family Desmacidonidae.



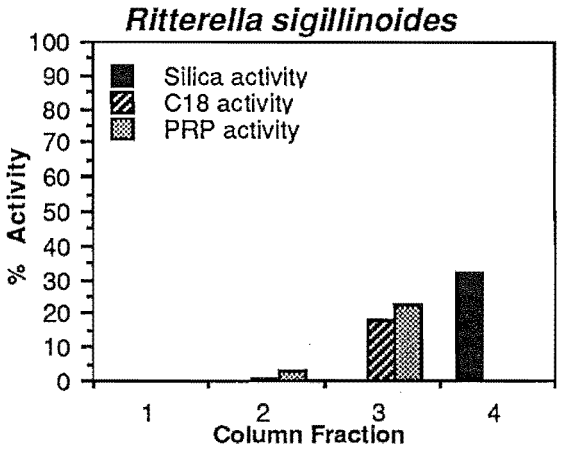
Antiviral/cytotoxicity assay:	?	?	ww	C9, C3	200
	ww	ww	3+	C1, C3	20
	2+	2+	1+	C3	5
	-	-	-		2
P388 assay (IC_{50}) (ng/ml):		45493			
Antimicrobial assay:	-	sm	-	-	200

Figure 7.5: Activity/chromatography profile of *Ircinia novaezelandiae*.



(212)

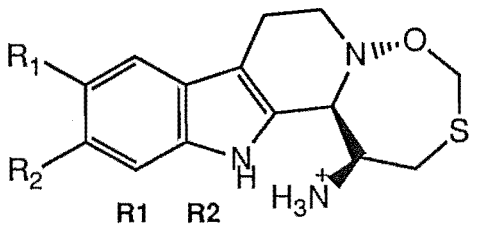
Variabilin (**212**), is the biologically active component of *Ircinia novaezelandiae* and is difficult to handle chromatographically, as it contains a non-polar tail but a polar head. This is reflected in the activity being in fractions 2 and 3 of the silica column and fraction 3 of the C18 reverse phase and phenyl reverse phase columns. Recovery of activity from the phenyl reverse phase column is slightly worse than that from the C18 reverse phase column, as the aromatic reverse phase material retains variabilin better than C18 reverse phase material. In all cases, recovery of activity is poor. For the C18 reverse phase and phenyl reverse phase columns, this is mainly because the standard profile of solvents used was too polar to elute much variabilin from the column. For the silica column, low recovery is due to strong absorption of the tetronic acid group on to the silica. If variabilin is methylated, recovery from silica is greatly improved[213].



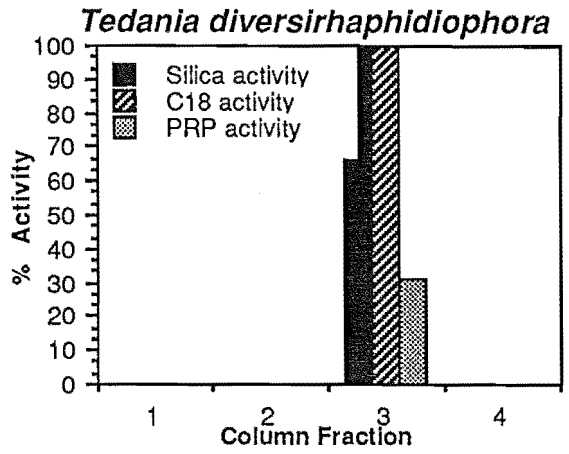
Antiviral/cytotoxicity assay:	ww	ww	±	C7*	5
	3+	2+	±	C7*	2
	-	-	-		0.5
P388 assay (IC_{50}) (ng/ml):		1424			
Antimicrobial assay:	-	sm	-	hm	200

Figure 7.6: Activity/chromatography profile of *Ritterella sigillinoides.*

A series of compounds of similar polarity to (**204**), the eudistomins (**61**), (**63**) and (**209**), are the biologically active components of *Ritterella sigillinoides.* Although the type of antiviral/cytotoxic activity is the same as that exhibited by the mycalamides (**210-211**), the activity/chromatography profile is different from those of both *Mycale* sp. I and *Strongylacidon* sp. I.



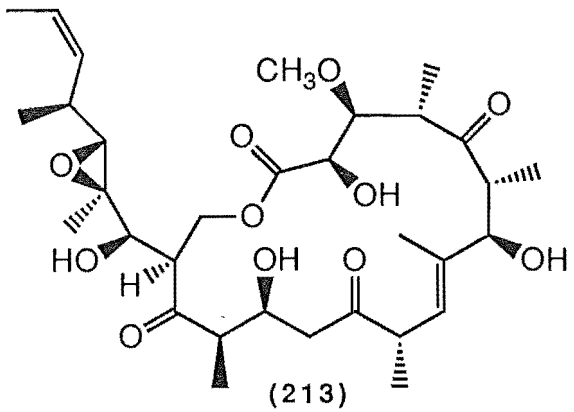
	R1	R2
(61)	OH	Br
(63)	H	Br
(209)	H	H



Antimicrobial assay:	-	-	-	-	200
Antiviral/cytotoxicity assay:	-	-	-		200
P388 assay (IC_{50}) (ng/ml):		854			
Antimicrobial assay:	-	-	-	-	200

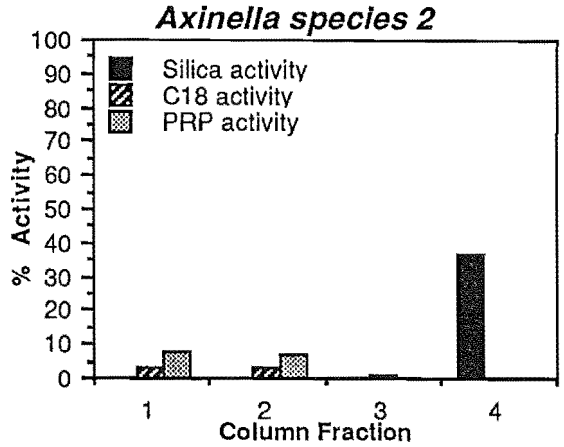
Figure 7.7: Activity/chromatography profile of *Tedania diversirhaphidiophora*

Tedania diversirhaphidiophora contains a very potent compound, tedanolide (**213**), but at a very low level, with the crude extract showing little antiviral/cytotoxic and P388 activity. The IC_{50} of the pure compound is reported as 16 pg/ml. This compound is quite non-polar as evidenced by its chromatographic behaviour, with biological activity concentrated in fraction 3 of each of the chromatography columns.



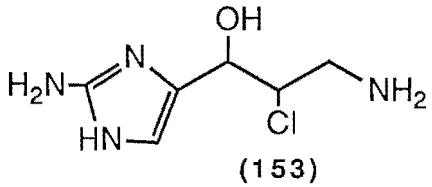
(213)

The screening method therefore provides a means to distinguish between the eudistomins, tedanolide and the mycalamides, although all give very similar results in the antiviral/cytotoxicity assay and have the same cytotoxicity type, C7*.

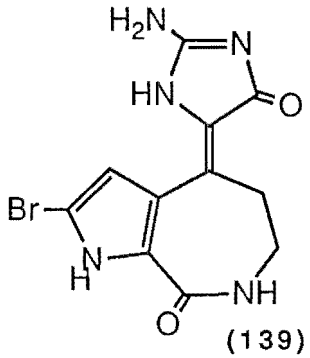


Antiviral/cytotoxicity assay:	ww	ww	±	C7*	50
	ww	ww	ww	C11	20
	-	-	ww	C11	5
	-	-	-		2
P388 assay (IC_{50}) (ng/ml):		3222			
Antimicrobial assay:	-	-	-	-	200

Figure 7.8: Activity/chromatography profile of *Axinella* sp. 2



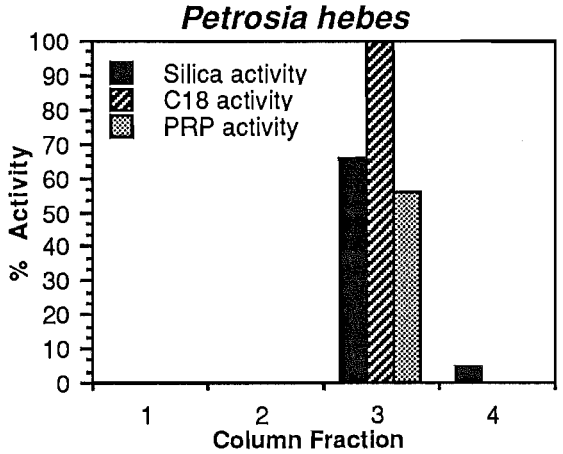
(153)



(139)

There are two active components in the sponge *Axinella* species 2, a very polar compound, girolline (**153**), and a more non-polar compound, hymenialdisine (**139**). The activity/chromatography profile for the extract reflects this, as do the assay results. This is the only standard extract for which activity is eluted in fraction 1 of the C18 reverse phase and phenyl reverse phase columns, emphasising the very polar nature of girolline. In the antiviral/cytotoxicity assay, girolline exhibits cytotoxicity type C7* while hymenialdisine typically exhibits cytotoxicity type C11. The assay results for the crude extract show this also, with C7* predominating at high concentrations and C11 with dilution. The reason for this is that there is much more hymenialdisine than girolline in the sponge but girolline is more potent. Assay results of column fractions show the two different cytotoxicity types also, with fraction 1 of the C18 reverse phase and phenyl reverse phase columns showing C7* and fractions 2 and 3 of the C18 reverse phase and fraction 2 of the phenyl reverse phase column showing

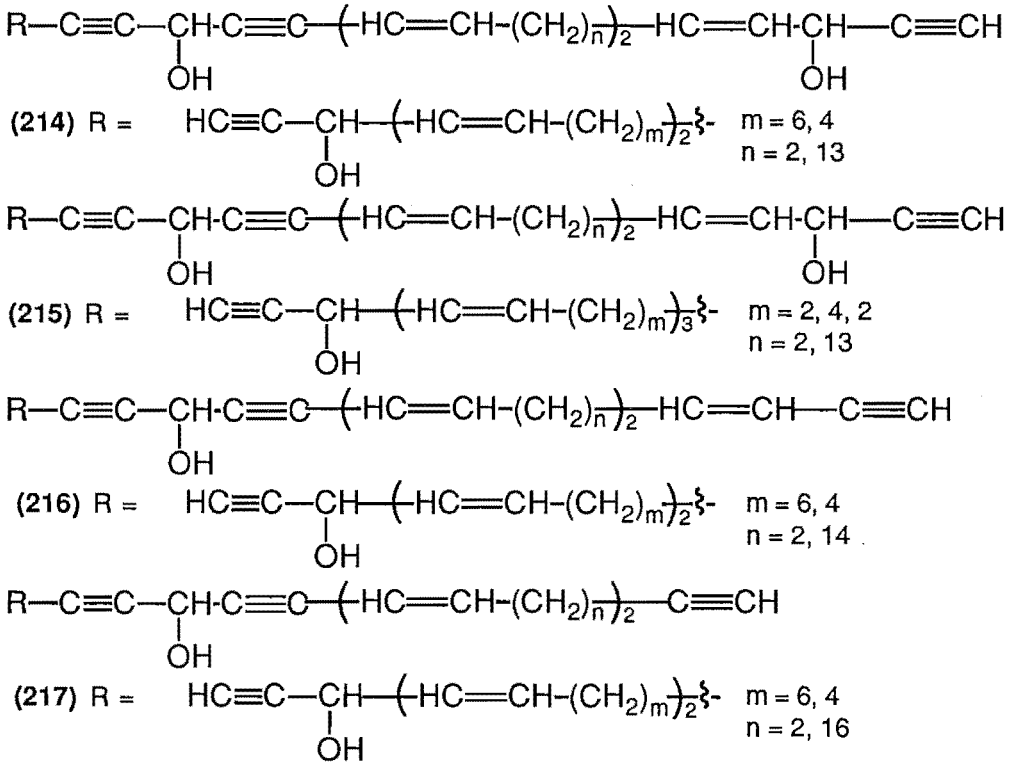
C11. Fraction 4 of the silica column shows C7* cytotoxicity, but recovery is low, as the solvents used are not polar enough to elute girolline. It would seem that hymenialdisine does not behave well on silica gel, indicating the unsuitability of silica gel column chromatography for this extract. Both girolline and hymenialdisine are active in the P388 assay, as indicated by every fraction of the reverse phase column showing activity in this assay. Girolline (**153**) is much more potent than hymenialdisine (**139**) with IC_{50} values of approximately 100 and 700 ng/ml respectively.

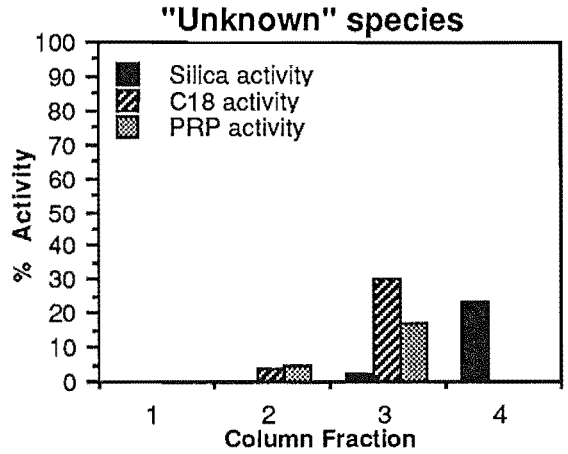


Antiviral/cytotoxicity assay:	3+	3+	±	C7	200
	-	-	-		50
P388 assay (IC_{50}) (ng/ml):		66178			
Antimicrobial assay:	-	-	-	-	200

Figure 7.9: Activity/chromatography profile of *Petrosia hebes*.

Petrosia hebes contains a number of polyacetylene compounds of the type (**214-217**) found in *Petrosia ficiformis*[214], which are responsible for the biological activity of the extract. These compounds are quite non-polar, as their chromatographic behaviour shows. Activity is concentrated in fraction 3 of both C18 reverse phase and phenyl reverse phase columns and mainly in fraction 3 of the silica column. These compounds are inactive in the antiviral/cytotoxicity assay system but quite strongly active in the P388 assay.



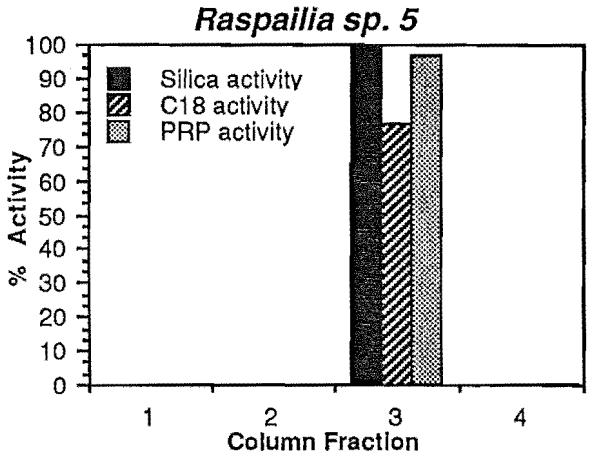


Antiviral/cytotoxicity assay:	ww	ww	±	C7	5
	2+	3+	±	C7	2
	-	-	-		0.5
P388 assay (IC_{50}) (ng/ml):		134			
Antimicrobial assay:	-	-	-	-	200

Figure 7.10: Activity/chromatography profile of "Unknown" extract.

The unknown extract had an activity/chromatography profile (Figure 7.10) that was remarkably similar to that obtained for *Mycale* sp. I and *Strongylacidon* sp. I. The active compounds in these species are the mycalamides (**210-211**). This "chemical screening" result suggested that the active component(s) in the unknown species could possibly also be the mycalamides. Subsequently, closer taxonomic

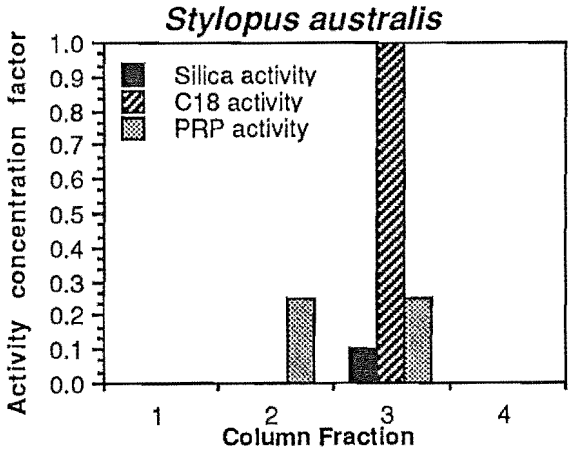
examination of this species showed it to also be *Mycale* sp. I, thereby demonstrating the value of the screening procedure.



Antiviral/cytotoxicity assay:	-	-	ww	C11	200
	-	-	2+	C11	50
P388 assay (IC_{50}) (ng/ml):		ND			
Antimicrobial assay:	-	-	-	-	200

Figure 7.11: Activity/chromatography profile of *Raspailia* sp. 5

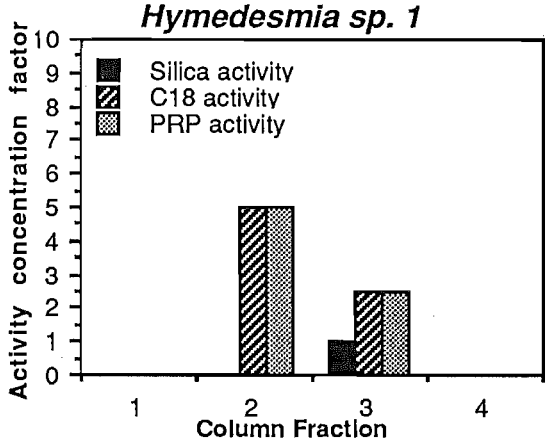
An extract of the sponge *Raspailia* sp. 5 was screened by the procedure, as the results of a survey of sponges of the order Axinellida indicated that it could contain hymenialdisine (See Chapter Four of this thesis). The biological assay results and the chromatographic behaviour of the biological activity are consistent with this possibility (Figure 7.11), although the recovery of activity from chromatography on *Raspailia* sp. 5 is much better than that from *Axinella* sp. 2 and ^1H nmr spectra of the active fractions did not contain any signals characteristic of hymenialdisine (**139**).



Antiviral/cytotoxicity assay:	-	-	-		200
P388 assay (IC_{50}) (ng/ml):		ND			
Antimicrobial assay:	-	-	-	-	200

Figure 7.12: Activity/chromatography profile of *Stylopus australis*

An extract of the sponge *Stylopus australis* was put through the screening procedure, and although the type of activity exhibited was similar to that of the mycalamides and the eudistomins, the chromatographic behaviour was distinctly different from that of these compounds (Figure 7.12), indicating that the active component is probably none of these.



Antiviral/cytotoxicity assay:	?	?	ww	C4,2,11	200
	±	±	2+	C4,2,11	50
	-	-	±	C3	20
	-	-	-		5
P388 assay (IC_{50}) (ng/ml):		ND			
Antimicrobial assay:	-	-	-	-	200

Figure 7.13: Activity/chromatography profile of *Hymedesmia* sp. 1.

An extract of the sponge *Hymedesmia* sp. I was also screened to gain some knowledge of the chromatographic behaviour of the biological activity. The results indicated that the active component is of medium polarity, but the activity/chromatography profile is again different from that of any of the standard extracts (Figure 7.13).

From the results, it is clear that the C18 reverse phase and phenyl reverse phase columns could well have had another fraction taken from them to extend the information they give on more non-polar compounds. Judging from experience of reverse phase chromatography on a larger scale, the fourth fraction could be either dichloromethane or methanol/dichloromethane (1:1).

EXTENSION TO ION EXCHANGE CHROMATOGRAPHY OF STANDARD EXTRACTS

The initial screening procedure has since been extended to cover the potential ion exchange properties of biologically active components in an extract. This ion exchange chromatography protocol has been developed by Dr. M. H. G.

Munro, for use in the National Cancer Institute's screening programme of natural product extracts. The procedure utilises both anion and cation exchange materials eluted with volatile buffers, to determine if the biologically active component of an extract is charged or neutral.

Ammonium acetate (0.025 M) was used as an equilibrating solvent for both anion and cation exchange resins. With a pH of approximately 6.5, use of this buffer ensures that most potential anions such as carboxyl, sulphate and sulphonate groups will be fully ionized and most amines will be protonated. Phenols are a major exception to this, as they would not be fully ionized at this pH. As an elution solvent, 0.5 M acetic acid was chosen for both anion and cation exchange resins. For anions, two types of interactions are possible. Weak anions may be protonated or the increased ionic strength of the 0.5 M acetic acid may elute anions from the resin. Similarly for cations, the increased ionic strength allows elution, and for a weak cation exhange resin, changing the pH removes the cation exchange properties to permit elution of cationic material. Sodium citrate (0.5 M) was chosen as an elution solvent for strong anions. As the citrate anion is selectively retained on the resin, it will displace all but very strongly retained anions such as sulphonate.

Aqueous natural product extracts were dry coated onto celite as previously described, to facilitate the loading of material onto the top of the resin column.

This procedure was utilised for the standard extracts in the current study with several modifications. As the extraction procedure used by the Marine Chemistry group at Canterbury at that stage did not involve obtaining both organic and aqueous extracts separately, solubility of the more lipophilic extracts in the buffers would be a problem. It was therefore decided to test only those extracts known to contain cationic or anionic biologically active compounds of an appropriate polarity in the system; namely those of *Latrunculia brevis*, *Axinella* sp. 2, and *Ritterella sigillinoides*. These extracts displayed significant biological activity in the first or second fractions from the C18 and phenyl reverse phase initial chromatography columns. For each extract, the most active fraction from these columns was used for the ion exchange chromatography, thereby preconcentrating the biological activity. Solvent systems used were the same as those outlined above, but the strong anion exchange resin SAX was used instead of DEA. Column fractions were assayed for biological activity in the antiviral/cytotoxicity assay system, as only the location of the biological activity

was required and not quantitative information. The results are summarised in Table 7.1.

Table 7.1: Results of ion-exchange chromatography on *Axinella* sp. 2, *Ritterella sigillinoides* and *Latrunculia brevis.*

Ion-exchange resin	Fraction number	Species		
		Axinella sp. 2	*Ritterella sigillinoides*	*Latrunculia brevis*
SAX	1	*	*	*
	2	* (slight)	-	-
	3	-	-	-
SCX	1	-	-	-
	2	-	-	-

* denotes that biological activity was detected in that fraction by the antiviral/cytotoxicity assay.
\- denotes no detectable biological activity.

The results for the standard extracts correspond well to the expected behaviour of the charged active components. Discorhabdin A (**204**), the eudistomins (**61**), (**63**) and (**209**), and girolline (**153**) all contain amine functionalities and would be expected to be retained on a cation exchange resin and to have no affinity for an anion exchange resin. This was indeed the case, with biological activity located in the first fraction from the anion exchange column for each extract, implying no affinity for the resin. No biological activity was recovered from the cation exchange column for each extract, implying that the active components of the extracts had a strong affinity for the resin.

GEL PERMEATION CHROMATOGRAPHY

The molecular weight or molecular weight range of the biologically active component of an extract is another very useful parameter in this type of work. Hplc gel permeation chromatography was attempted in an effort to obtain this information.

The gel permeation column used was an hplc analytical column, packed with PL gel, a highly cross-linked divinylbenzene. The pore size of 50 Å was the smallest available and it was hoped that it would be suitable for use with compounds in the molecular weight range of approximately one hundred to one thousand daltons. Acetonitrile was chosen as a universal elution solvent, as it enables uv detection to

be carried out at low wavelengths and is a good solvent of medium range polarity, so most of the active components of the standard extracts should have been soluble in it.

The test mixture of toluene in acetonitrile was injected onto the column and gave a well shaped peak of reproducible elution volume when monitored by uv detection. Calibration of the column was then attempted using compounds of varying molecular weights: namely polyethylene glycol, napthalene and toluene. Napthalene gave spurious results, probably on account of having a high affinity for the gel used, so mycalamide A (**210**) was substituted and a calibration curve (Figure 7.14) constructed to determine the column volume.

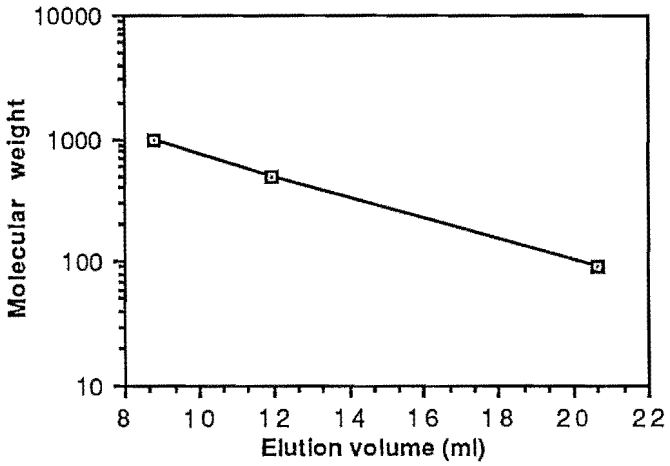


Figure 7.14: Calibration curve for PL gel column in acetonitrile.

Gel permeation chromatography was attempted, both on some pure active compounds and on some column fractions from the initial screening of the standard active extracts. Samples tested were mycalamide A (**210**), discorhabdin C (**206**), the TFA salt of ritterellin I (**61**), variabilin (**212**) and active column fractions from the initial chromatography columns on *Mycale* sp. I, *Latrunculia brevis*, *Ircinia novaezelandiae*, *Tedania diversirhaphidiophora*, *Strongylacidon* sp. I and *Petrosia hebes*.

For each run, eight fractions were collected across a total of three column volumes of solvent. These fractions covered the molecular weight range of > 1000 to 100 assuming that no absorption was taking place on the column. The fractions were initially submitted for P388 assay at constant volume in acetonitrile, but solvent blanks proved this to be too cytotoxic, so fractions were dried down and submitted for assay in methanol instead.

The results for both the pure compounds and the column fractions are summarised in Table 7.2.

Table 7.2: Results of gel permeation chromatography in acetonitrile for pure compounds and partially purified crude extracts.

	Fractions							
	MW>1000		MW 500					MW<100
	1	2	3	4	5	6	7	8
Pure Compounds (MW)								
mycalamide A (503)	-	-	*	*	-	-	-	-
discorhabdin C (464)	-	-	-	*	-	-	-	-
ritterellin I TFA salt (356)	-	-	-	-	-	-	-	-
variabilin (398)	-	-	-	-	-	-	-	-
Semi-crude extracts								
T. diversirhaphidiophora (640)	*	*	-	-	-	-	-	-
Strongylacidon sp. (503)	-	-	*	-	-	-	-	-
Mycale sp. I (503)	-	-	*	*	*	-	-	-
Petrosia hebes (668)	-	-	*	-	-	-	-	-
Latrunculia brevis (427)	-	-	-	-	*	-	-	-
I. novaezelandiae (398)	*	*	*	*	*	*	*	*

* denotes that activity was detected in that fraction.

For mycalamide A (**210**), biological activity was concentrated in fraction 4 with an IC_{50} of 854 ng/ml, although some biological activity was detected in fraction 3 (4915 ng/ml). This activity coincided with the weak uv peak detected at 200 nm and was in the same region in which the mycalamide A in the standard sample was eluted.

When a sample of red discorhabdin C (**206**) was injected onto the column, no peak was detected at the λ_{max} of 260 nm and no colour was visible in the column effluent, indicating that the compound did not come off the column. When a sample was then injected onto the column using acetonitrile to which 0.5% glacial acetic acid by volume had been added, uv detection indicated that the compound was now eluting from the column and at approximately the same retention time as observed for mycalamide A.

A sample of ritterellin I TFA salt however, did not elute from the column, either with acetonitrile, or when glacial acetic acid was added.

Variabilin was the other pure compound tested and it appeared to be eluting from the column, but gave a surprisingly weak response by uv detection.

The results for the column fractions of extracts were less encouraging. When a column fraction of the *Tedania diversirhaphidiophora* extract was injected onto the column, most of the biological activity was concentrated in the first fraction collected (518 ng/ml), with some activity in fraction 2, a result that was not consistent with the molecular weight of the active component, tedanolide.

For a sample of semi-purified *Strongylacidon* extract, the biological activity was detected in fraction 3 from the gel permeation column (2441 ng/ml), not in fraction 4 as would be expected from the results obtained on pure mycalamide A. For a *Mycale* sp. I sample, most of the activity was concentrated in fraction 4 from the column (141 ng/ml) with some detected in the fractions on either side, fractions 3 and 5 with IC_{50} values of 1339 and 5167 ng/ml respectively.

Biological activity was detected in fraction 3 for a *Petrosia hebes* sample, with an IC_{50} of 4915 ng/ml. This is a greater elution volume than expected as the estimated molecular weights of the active compounds are 666 and 668.

The *Latrunculia brevis* sample was run with acetic acid in the acetonitrile and weak activity was detected in fraction 5 from the column, with an IC_{50} of 10938 ng/ml.

When a sample of *Ircinia novaezelandiae* was injected onto the column, traces of biological activity were detected by the antiviral/cytotoxicity assay throughout the column fractions. However, large uv absorption peaks were detected for up to fifty minutes after injection of the sample, showing that the compounds in the extract are strongly retained by the gel.

A plot of the logarithm of the molecular weight of the active compounds versus their actual elution volume, is shown in Figure 7.15. As this graph shows, the correspondence between molecular weight and elution volume is rather poor, with some compounds leaching off the column over a wide volume range.

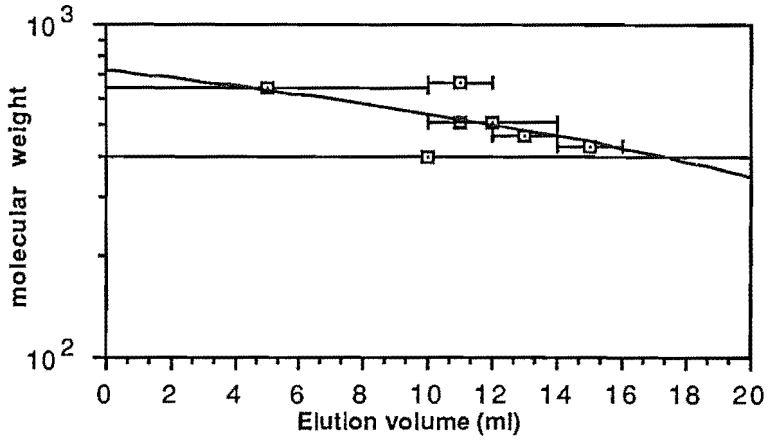


Figure 7.15: Results of gel permeation chromatography in acetonitrile for pure compounds and partially purified extracts.

It was then decided to attempt calibration of the column in methanol, as the fractions could be submitted directly for assay without being dried down, and the possibility existed when using acetonitrile that traces of solvent were interfering with the assays in some cases. It was thought best to calibrate the column with a number of compounds of varying molecular weights but with strong uv chromophores. Compounds chosen were toluene (molecular weight (MW) =92), napthalene (MW=128), 1,4-dibromonapthalene (MW=288), bromothymol purple (MW=394), bromocresol purple (MW=540), bromocresol green (MW=698) and disulphine blue (MW=1158). For each compound, the wavelength at which maximum absorption of uv light occurred was determined by running an uv absorption spectrum and uv detection was then at the appropriate wavelength for each compound injected separately onto the column. The difference in retention time on the column between bromocresol green, bromocresol purple and bromothymol purple was less than 0.5 minute. Disulphine blue had a longer retention time than these three compounds, though it is of higher molecular weight. This is possibly a consequence of it being a charged compound.

Toluene in methanol had a longer retention time on the column than in acetonitrile, yet other compounds appeared to have shorter retention times than would have been expected in acetonitrile, according to their molecular weight.

Neither napthalene nor 1,4-dibromonapthalene seemed to elute from the column at all, supporting the observation that the gel must have high affinity for aromatics of this kind.

In light of the above results, use of the PL gel column for screening extracts was abandoned. The main problems with the use of this column were its high affinity for some aromatic compounds, its lack of clear differentiation of molecular weights between one hundred and one thousand and its intolerance to water in the solvent system, thus precluding the screening of some compounds on the grounds of solubility.

If gel permeation is to be used in the screening of extracts in future, some modifications would be appropriate. The amount of material that can be loaded onto a gel permeation column is much lower than that which can be loaded onto a reverse phase or normal phase chromatography column, with a loading ratio of only one to one thousand. Thus sensitivity may be a problem when dealing with crude extract. Also, the use of one universal solvent is not particularly convenient, as there will always be compounds at either end of the polarity scale not soluble in a solvent of medium polarity and those of low solubility which may require a special solvent system. Therefore, for both solubility and sensitivity reasons, it would seem to be more convenient to use an active column fraction from the initial chromatography columns run on an extract for gel permeation attempts, rather than crude extract. In this way, the activity is preconcentrated to enhance its detection in the fractions collected and the polarity of the active component is known. With this information, a column of gel permeation material such as Fractogel PGM 2000 for example, can be made up in the solvent most suitable to the polarity of the active component and calibrated with known compounds. Fractions could be assayed for biological activity in the usual way.

Thus it is recommended that both gel permeation and ion exchange chromatography be used as a secondary screen only as appropriate, after the initial screening by reverse phase and normal phase chromatography of the crude extract.

MODIFICATIONS OF THE SCREENING PROCEDURE

A modification of the procedure used in these initial studies, is now used routinely by members of the Canterbury Marine Chemistry group to assess the suitability of an extract for further large scale work and to determine the probability of whether the compound(s) under study are known or are novel structures. This now involves the use of commercially available cartridges of chromatographic stationary

phases and solvent systems similar to those described above, to assist in the selection of extracts for research.

As the procedure has been used by the Canterbury group, it has undergone further modifications through constant critical evaluation of the information gained from the results. The procedure could obviously be adapted according to the requirements of any research group, depending on such factors as whether organic and aqueous extractions are performed separately and the range of chromatographic stationary phases and biological assays available. The most recent version of the procedure, developed by Dr. Murray Munro, involves obtaining both aqueous and organic extracts separately.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES

Melting points were obtained using an Electrothermal melting point apparatus and were uncorrected. Optical rotations were measured for methanol solutions using a Jasco J-20c automatic recording spectropolarimeter. Fab mass spectra were obtained on a VG7070F spectrometer, EI/CI mass spectra were obtained on a Kratos MS80RFA mass spectrometer, while lcms data were obtained from a Finnigan TSP46 spectrometer equipped with a thermospray source. SIMS mass spectra were obtained on a Hitachi M-80B double focusing mass spectrometer equipped with a primary ion (Xe) source. Gcms was performed on a Hewlett Packard HP-Ultra 2 capillary column, cross-linked 5% phenyl methyl silicone (50 m $\times$ 0.32 mm $\times$ 0.52 μm), using a Hewlett Packard 5710A gas chromatograph linked to a Hitachi M-80B mass spectrometer equipped with a M-0101 data system. Samples were run under a temperature programme from 200-310°C at 1° per minute with a split ratio of 25:1. Ir spectra using either KBr discs or smears in chloroform or methanol, were obtained using a Pye Unicam SP-300 infrared spectrophotometer with the 1603 cm^{-1} band of polystyrene as a reference. Uv spectra were obtained as methanol solutions using a Varian DMS 100 spectrophotometer.

^{13}C nmr spectra at 75 MHz and ^{1}H nmr spectra at 300 MHz were recorded on a Varian XL-300 spectrometer. Chemical shifts are expressed as parts per million (ppm) relative to the solvent reference used: $CDCl_3$/TMS: ^{1}H 7.25 ppm. ^{13}C 77.01 ppm; CD_3OD: ^{1}H 3.30 ppm, ^{13}C 49.30 ppm; D_2O: ^{1}H 4.70 ppm, ^{13}C 67.40 ppm (dioxane as internal reference); d6-DMSO: ^{1}H 2.60 ppm, ^{13}C 39.60 ppm; C_6D_6: ^{1}H 7.27 ppm. The shifts are quoted as position (δ), relative integral, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and br, broad), coupling constant (J, Hz) and the assigned atom label.

Difference nuclear Overhauser effect (nOe) spectra were obtained in an arrayed experiment with the decoupler offset 10 000 Hz for the control experiment and subsequently cycled over the multiplet peaks of each proton to be irradiated[215]. Two dimensional correlation spectra were recorded on the XL 300 spectrometer in the usual manner[216,217], using standard software. All data were displayed in the absolute value mode.

Flash column chromatography was performed using silica gel (Davisil 35-70 μ) and C18 reverse phase material prepared by coating the silica gel with n-octadecyltrichlorosilane after the method of Evans *et al* [218]. Columns were generally run under nitrogen pressure. Fractogel PGM 2000 (Merck) was used for gel permeation chromatography. Medium pressure liquid chromatography (mplc) was performed using a Milton-Royal pump, Rheodyne type 50 injector and a LKB 2212 Helirac fraction collector on a Lobar diol column (Merck LiChroprep), with detection by means of a LKB 2142 differential refractometer and a LKB 2238 Uvicord II uv detector.

High pressure liquid chromatography (hplc) was performed on a Varian 5000 liquid chromatograph using a Varian UV-50 variable wavelength detector and a Hewlett Packard 3390A integrating recorder. Semi-preparative reverse phase chromatography was carried out on either Alltech C8, C18 or Cyanopropyl (CN) columns (250 x 10 mm) or on a Rainin Dynamax C18 column (250 x 21.4 mm). Analytical reverse phase chromatography was performed on an Alltech C18 column (250 x 4.6 mm).

All solvents used were either spectral grade or distilled prior to use.

WORK DESCRIBED IN CHAPTER TWO

2.2 STUDIES OF THE BRYOZOAN, *CRIBRICELLINA CRIBRARIA*

COLLECTION AND EXTRACTION OF BRYOZOAN

Cribricellina cribraria (1 kg wet) was collected by SCUBA diving in April 1987 from Sugar Loaf, Kaikoura, off the South Island of New Zealand. A voucher specimen, 87K15-02, is held at the Chemistry Department, University of Canterbury. The bryozoan was stored frozen, then in a typical extraction, 200 g was left overnight in methanol/toluene 4:1, (250 ml) and blended three times in the same solvent mixture. The combined extracts were filtered under vacuum, the filtrates combined and the solvent removed under reduced pressure.

1-ETHENYL-9H-PYRIDO-[3,4-b]INDOL-8-OL (1-VINYL-8-HYDROXY-β-CARBOLINE) (**82**)

The resulting extract (7.6 g) was chromatographed on a reverse phase C18 column (50 g), using a water/methanol to methanol/dichloromethane gradient to give 13 fractions. Alkaloids were shown to be concentrated in fractions 6 and 7 from the

reverse phase column by tlc (ethyl acetate: acetone (1:1), fluorescent spots under uv light) and by P388 assay. These two fractions (116 mg) were recombined and rechromatographed on a reverse phase C18 column (10 g) using the same water/methanol/dichloromethane gradient as previously. Alkaloids were concentrated in fraction 4 by tlc and P388 assay.

A further reverse phase C18 column on fraction 4 (37 mg) led to the isolation of 1-vinyl-8-hydroxy-β-carboline (**82**) (20 mg) as a yellow oil. For the isolation scheme used, refer to Figure A.1 in the Appendix.

ms m/z found : $M^{+\cdot}$ 210.0787 (4), ($C_{13}H_{10}N_2O$ requires $M^{+\cdot}$ 210.0793), 209.0702 (2), $C_{13}H_9N_2O$ requires 209.0715), 184.0636 (0.4), ($C_{11}H_8N_2O$ requires 184.0637); UV λ_{max} (MeOH) 369, 296, 244, 230 nm (log ε 3.93, 3.97, 4.09, 4.06 respectively), λ_{max} (NaOH-MeOH) 386, 287, 262, 240 nm (log ε 3.82, 4.03, 4.06, 4.07 respectively); ir ν_{max} (smear) 3590 br, 3140, 2990, 1630, 1570, 1510, 1480, 1430, 1280, 1230, 780 cm^{-1}. For ^1H and ^{13}C nmr spectral data, refer to Tables 2.1 and 2.2.

1-ETHENYL-8-METHOXY-9H-PYRIDO-[3,4-b] INDOLE
(1-VINYL-8-METHOXY-β-CARBOLINE) (**88**)

1-vinyl-8-hydroxy-β-carboline (**82**) (15 mg) was dissolved in methanol (1 ml). Diazomethane in ether was added to the sample, which was left for 3 hours. After this time, tlc and ^1H nmr spectroscopic analysis showed the formation of a complex mixture but with 1-vinyl-8-methoxy-β-carboline (**88**) as the major component. This was purified by reverse phase column chromatography to yield 4 mg of a yellow-green oil.

ms m/z found $M^{+\cdot}$ 224.0949 (76), ($C_{14}H_{12}N_2O$ requires $M^{+\cdot}$ 224.0949), 223.0848 (35), ($C_{14}H_{11}N_2O$ requires 223.0872) 209.0729 (45), ($C_{13}H_9N_2O$ requires 209.0715), 194.0832 (13), ($C_{13}H_{10}N_2$ requires 194.0844); UV λ_{max} (MeOH) 363, 285, 267, 249, 220 nm (log ε 3.23, 3.43, 3.69, 3.93, 3.96 respectively); ir ν_{max} (smear) 3550, 3050, 2925, 1625, 1575, 1420, 1310, 1260, 1240, 1050 cm^{-1}. For ^1H and ^{13}C nmr spectral data refer to Tables 2.1 and 2.2. $^1J_{CH}$ (HETCOR) H3 <-> $C_{139.19}$, H4 <-> $C_{114.32}$, H5 <-> $C_{113.86}$, H6 <-> $C_{120.78}$, H7 <-> $C_{107.97}$, H1' <-> $C_{132.98}$, H2'a, H2'b <-> $C_{119.09}$, H8' <-> $C_{55.62}$; $^2J_{CH}$, $^3J_{CH}$ (HMBC) H3 <-> C4, H4 <-> C4b, H5 <-> C4a, C4b, C7, C8a, H6 <-> C4b, C8, H7 <-> C5, C8, C8a, H1' <-> C1, C4a, H2'a <-> C1, C1', H2'b <-> C1, H8' <-> C8, C8a.

1-ETHYL-9H-PYRIDO[3,4-b]INDOL-8-OL
(1-ETHYL-8-HYDROXY-β-CARBOLINE) (**89**)

1-vinyl-8-hydroxy-β-carboline (**82**) (4 mg) in methanol (1 ml) was stirred with platinum dioxide (0.5 mg) under hydrogen until absorption ceased. After filtration of the solution through a bed of celite in methanol to remove the catalyst, 1-ethyl-8-hydroxy-β-carboline (**89**) (4 mg) was isolated as a pale yellow oil.
ms m/z found $M^{+\cdot}$ 212.0948 (100), ($C_{13}H_{12}N_2O$ requires $M^{+\cdot}$ 212.0950), 211.0865 (99), ($C_{13}H_{11}N_2O$ requires 211.0871), 184.0647 (17), ($C_{11}H_8N_2O$ requires 184.0636), UV λ_{max} (MeOH) 341, 287, 278, 243, 219 nm (log ε 2.98, 3.25, 3.20, 3.88, 3.52 respectively); λ_{max} (NaOH-MeOH) 358, 288, 279, 253, 226 nm (log ε 2.91, 3.18, 3.26, 3.77, 3.64 respectively); ir ν_{max} (smear) 3390 br, 3005, 2950, 1640, 1590, 1490, 1440, 1385, 1245, 1230 cm^{-1}. For ^1H and ^{13}C nmr spectral data refer to Tables 2.1 and 2.2. $^2J_{CH}$, $^3J_{CH}$ (HMBC) H3 <-> C1, C4, C4a, H4 <-> C3, C4a, H5 <-> C4a, C7, C8a, H6 <-> C4b, C8, H7 <-> C5, C8a, H1' <-> C1, C4a, C2', H2' <-> C1, C1'.

1-ETHENYL-8-ACETOXY-9H-PYRIDO-[3,4-b] INDOLE
(1-VINYL-8-ACETOXY-β-CARBOLINE) (**90**)

1-vinyl-8-hydroxy-β-carboline (**82**) (5 mg) was dissolved in pyridine/acetic anhydride (1:1) (1 ml) and left stirring overnight. Solvent was removed and 2 ml of water was added. Extraction with ethyl acetate (3 x 1 ml) led to the isolation of 3 mg of (**90**) as a yellow oil.
ms m/z found $M^{+\cdot}$ 252.0895 (35), ($C_{15}H_{12}N_2O_2$ requires $M^{+\cdot}$ 252.0899), 210.0806 (100), ($C_{13}H_{10}N_2$ requires 210.0794), 195.0910 (17), ($C_{13}H_{11}N_2$ requires 195.0923), 168.0655 (14), ($C_{11}H_8N_2$ requires 168.0688); UV λ_{max} (MeOH) 349, 298 sh, 287, 272, 244, 221 nm (log ε 2.79, 2.91, 3.13, 3.19, 3.57, 3.58 respectively), UV λ_{max} (NaOH-MeOH) 373, 288, 254, 237, 233 nm (log ε 2.61, 3.18, 3.45, 3.54, 3.54 respectively); ir ν_{max} (smear) 3370, 2950, 1740, 1630, 1590, 1440, 1270, 1240, 1210, 1105, 1040, 800 cm^{-1}. For ^1H and ^{13}C nmr spectral data, refer to Tables 2.1 and 2.2. $^1J_{CH}$ (HETCOR) H3 <-> $C_{139.48}$, H4 <-> $C_{115.82}$, H5 <-> $C_{121.45}$, H6 <-> $C_{120.63}$, H7 <-> $C_{122.54}$, H1' <-> $C_{132.64}$, H2'a <-> $C_{120.08}$, H2'b <-> $C_{120.08}$, H8' <-> $C_{21.26}$.

1-ETHYL-8-METHOXY-9H-PYRIDO-[3,4-b] INDOLE
(1-ETHYL-8-METHOXY-β-CARBOLINE) (**91**)

1-vinyl-8-methoxy-β-carboline (**88**) (2 mg) in methanol (1 ml), was stirred with platinum dioxide (0.5 mg) under hydrogen for 30 minutes. Tlc analysis (ethyl

acetate/acetone (1:1), fluorescent spot under uv light) indicated that a single product of lower Rf had formed. After filtration of the solution through a bed of celite in methanol to remove the catalyst, 1-ethyl-8-methoxy-β-carboline (**91**) was isolated as a pale yellow oil, the ^1H nmr and mass spectra of which, were consistent with literature values[68].

1-ETHYL-4-METHYL-SULPHONE-β-CARBOLINE (**92**)

Fractions from the flash reverse phase columns on the extract were shown to contain a component, fluorescent under uv light at Rf = 0.9 on tlc (silica, ethyl acetate/acetone, (1:1)). Reverse phase hplc of these fractions, using an Alltech cyanopropyl semi-preparative column, flow rate 5 ml/minute, methanol/water (2:3) and uv detection at 220 nm, led to the isolation of 4 mg of (**92**) as a pale green oil. ms m/z found $M^{+\cdot}$, 274.0777 (100), ($C_{14}H_{14}N_2O_2S$ requires $M^{+\cdot}$ 274.0776), 246.0470 (12), ($C_{12}H_{10}N_2O_2S$ requires 246.0463), 195.0931 (13), ($C_{13}H_{11}N_2$ requires 195.0922), 168.0734 (7), ($C_{11}H_8N_2$ requires 168.0687); UV λ_{max} (MeOH) 350, 304, 299, 258, 250, 213 nm (log ε 2.62, 3.03, 2.94, 3.32, 3.29, 3.43 respectively); ir ν_{max} (smear) 3390, 2990, 1650, 1600, 1570, 1485, 1430, 1335, 1160, 1010, 785 cm^{-1}. For ^1H and ^{13}C nmr spectral data refer to Table 3.4.

ISOLATION OF HARMAN (**55**)

A fraction (21 mg) from the second reverse phase chromatography column on the extract, was subjected to further chromatography on C18 reverse phase material using a water to methanol gradient to yield 15 fractions. Tlc analysis revealed a spot, fluorescent under uv light to be present in several fractions and fractions 9 and 10 from the column were clean in this component by ^1H nmr spectroscopy in $CDCl_3$. These were combined to give a yellow oil (6 mg), the ^1H, ^{13}C nmr and mass spectra of which were identical with literature values for harman[65,76].

IDENTIFICATION OF 1-ETHYL-β-CARBOLINE (**56**)

Some of the column fractions containing harman by tlc analysis, contained another spot at higher Rf on silica gel, also fluorescent under uv light. The ^1H nmr spectra of these fractions in $CDCl_3$ showed no additional signals to those of harman, apart from a mutually coupled quartet and triplet ascribed to an ethyl group. This component had the same Rf value on silica gel as a synthetic sample of 1-ethyl-β-

carboline and the mass spectrum was identical to that reported in the literature for 1-ethyl-β-carboline[69].

ISOLATION OF PAVETTINE (**57**)

Signals characteristic of a vinyl group as in compound (**82**) were seen in the ^1H nmr spectrum in $CDCl_3$ of one column fraction (2 mg) which also contained harman and 1-ethyl-β-carboline. The sample was dissolved in chloroform and streaked along the baseline of a silica gel tlc plate (6 x 10 cm). The plate was developed in ethyl acetate/acetone (1:1) and visualised under uv light. The uv active band at highest Rf was scraped off the plate, the silica eluted with ethyl acetate/acetone and filtered to yield <1 mg of a yellow-green oil. This was identified as pavettine from its ^1H nmr and mass spectra[77] and by tlc comparison with an authentic sample.

HOMARINE (**94**)

Fraction 2 from the initial reverse phase chromatography column on the crude extract, contained a pale lemon precipitate (117 mg), which was identified as homarine by ^{13}C nmr spectroscopy[78].

STEROLS

Fraction 8 from the initial reverse phase chromatography column on the extract (141 mg) was further partitioned by column chromatography on silica gel, using a petroleum ether to ethyl acetate gradient to yield 10 fractions. Fraction 5 from the column (19 mg) was determined to contain cholest-4-en-3-one (**107**) as a major component by ^{13}C nmr spectroscopy[81]. Fraction 7 from the column (82 mg) was determined to contain predominantly cholesterol (**101**) by ^{13}C nmr spectroscopy[81], while fraction 6 (25 mg) was a mixture of sterols. Fractions 5-7 were combined (127 mg) and a sub-sample of this mixture (55 mg) sent to Professor Carl Djerassi for analysis by gcms.

2.3 STUDIES OF THE BRYOZOAN, *MARGARETTA BARBATA*

BIOLOGICAL ACTIVITY OF *MARGARETTA BARBATA*

Fraction CB5 8.6 (211 mg), originally from a reverse phase column of a crude extract of *Margaretta barbata* (87K16-01)[79], was reassayed in the three biological assay systems and ^{1}H and ^{13}C nmr spectra obtained in $CDCl_3$. Analytical hplc was performed on this fraction, using an Alltech cyanopropyl column and a flow rate of 1 ml per minute in methanol/water (3:2) with uv detection at 220 nm. Antiviral/cytotoxicity assay of the four resulting cuts revealed that activity was spread throughout the fractions.

A sub-sample of the original material (28 mg) was taken up in ethanol/water (19:1) (5 ml) and extracted with petroleum ether (3 x 20 ml), then back extracted with ethanol/water (10 ml). Tlc analysis and biological assay of the resulting partitions showed that this was unsuccessful and the fractions were recombined.

Reverse phase flash column chromatography using C18 material (10 g) and a water to methanol to dichloromethane gradient was performed on the semi-crude material (135 mg) and 13 fractions were taken. Fractions 1-3 were recombined (58 mg) and further partitioned by reverse phase column chromatography on C18 material (10 g) using a similar solvent gradient, to yield 11 fractions. P388 assay showed that the biological activity was concentrated in fraction 6 from this column (17 mg). A ^{1}H nmr spectrum of this fraction in $CDCl_3$ was broad and unresolved.

Methylation of a sub-sample of this fraction (0.5 mg) with diazomethane in ether did not alter the biological activity so the whole sample (16.5 mg) was methylated by the same procedure and submitted to reverse phase hplc using a Rainin Dynamax C18 column and a flow rate of 10 ml per minute in methanol/water (9:1) with uv detection at 220 nm to yield 6 fractions. Biological activity was concentrated in fractions 2 and 3 by P388 assay, so these were combined (3 mg). The ^{1}H nmr spectrum of these combined fractions in $CDCl_3$ was again broad and unresolved and addition of a drop of TFA failed to sharpen the signals. The residue that was insoluble in $CDCl_3$ (1 mg) was checked by P388 assay and found to be inactive, so 1.8 mg of active material remained. Further work on this sample was abandoned due to lack of material.

Tlc analysis of fraction 5 (16 mg) from the second reverse phase column on CB 5 8.6 in ethyl acetate/acetone (1:1), revealed a number of spots, fluorescent under uv light and the ^{1}H nmr spectrum of this fraction in $CDCl_3$ revealed signals

reminiscent of a β-carboline nucleus. Semi-preparative reverse phase hplc of this fraction, using an Alltech cyanopropyl column and a flow rate of 5 ml per minute in methanol/water-TFA (3:2) with uv detection at 220 nm, led to the isolation of a yellow oil, the ^{1}H and ^{13}C nmr, uv and mass spectra of which, were consistent with those reported for harman (**55**)[65,76]. Another fraction containing less than 1 mg of material, was determined to contain harman and 1-ethyl-β-carboline (**56**) by mass spectrometry.

WORK DESCRIBED IN CHAPTER THREE

SYNTHESES OF β-CARBOLINE ALKALOIDS
GENERAL SYNTHETIC METHOD[77,88].

In a typical preparation, 500 mg of commercial L-tryptophan was dissolved in water (125 ml) to which 0.5 ml concentrated sulphuric acid had been added. 1-2 ml of freshly distilled aldehyde was added and the solution refluxed for ten minutes. It was then removed from the heat and 25 ml of a 10% potassium dichromate solution slowly added. The solution was left at room temperature for five minutes then reboiled and allowed to cool. Sulphur dioxide gas was bubbled into the solution or solid sodium sulphite added to destroy the excess oxidising agent. The solution was made basic by addition of solid sodium carbonate and extracted with dichloromethane (4 x 75 ml). The organic partition was dried over sodium sulphate, filtered and the solvent removed. The resulting crude product was purified by reverse phase flash column chromatography on C18 material using a water to methanol gradient. Column fractions were analysed by tlc and ^{1}H nmr spectroscopy in $CDCl_3$. Those fractions that contained the pure β-carboline alkaloids were combined and used for further nmr spectroscopic experiments and for biological assays.

NORHARMAN (**58**)

Yield: 7%. Recrystallised as colourless needles from petroleum ether/ethyl acetate. m.p.198°C, lit. 199-201°C[219]. The ^{1}H nmr[220], ^{13}C nmr[65] and uv[94] spectra were consistent with literature values.

1-ETHYL-β-CARBOLINE (**56**)

Yield: 12%. Recrystallised from chloroform/methanol to yield a small quantity of clear, cubic crystals (10 mg). m.p. 194°C, lit. 194-195°C[221]. ^{1}H nmr[69], ^{13}C nmr[65], uv[69] and mass spectra[69] were consistent with literature values.

1-PROPYL-β-CARBOLINE (**113**)

Yield: 15%. The uv[87], ir and mass spectra[83] were consistent with literature data.

1-ISOPROPYL-β-CARBOLINE (**114**)

Yield: 9%. The ir and mass spectra were consistent with literature data[83].

1-HEXYL-β-CARBOLINE (**115**) AND 1-PHENYL-β-CARBOLINE (**116**)

A modification of the above procedure was used. Tryptophan (500 mg) was dissolved in water (30 ml) to which concentrated sulphuric acid (0.1 ml) had been added. 1-2 ml of freshly distilled aldehyde was added and the solution left to reflux with vigorous stirring. The reflux period was overnight for 1-hexyl-β-carboline and four hours for 1-phenyl-β-carboline. The remainder of the preparation procedure was carried out as above.

1-HEXYL-β-CARBOLINE (**115**)

Yield: 1% of a clear oil. The uv and mass spectra were consistent with literature data[87].

1-PHENYL-β-CARBOLINE (**116**)

Yield: 19%. Uv and ir spectra were consistent with literature values[94].

1-METHYL-1,2,3,4-TETRAHYDRO-β-CARBOLINE-3-CARBOXYLIC ACID (**117**)[89]

L-tryptophan (300 mg) was dissolved in hot water (10 ml). The solution was cooled and 1 ml of freshly distilled acetaldehyde added with stirring. The vessel was loosely capped and the solution left stirring overnight at room temperature. A thick white precipitate was formed and removal of solvent under reduced pressure yielded 350 mg of crude product which was purified by reverse phase flash column chromatography on C18 material to give 198 mg of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**117**) as a white powder. Yield: 51%. The mass spectrum was consistent with literature data[82].

CONVERSION OF (**117**) TO HARMAN

1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**117**) (145 mg) was dissolved in water (40 ml) and refluxed. A 10% potassium dichromate solution (7.5 ml) and acetic acid (1.5 ml) were added and the solution refluxed for one minute longer, then cooled. 1 M sodium sulphite solution (100 ml) was slowly added, then the solution made basic by addition of solid sodium carbonate. Extraction with dichloromethane (3 x 75 ml) then ethyl acetate (3 x 75 ml), drying over sodium sulphate, filtering and removal of solvent gave 69 mg of semicrystalline harman which was purified by reverse phase column chromatography. Yield : 60%.

A second preparation of harman by this method without purification of the acid precursor (**117**) resulted in a 55% yield of harman. The ^{1}H nmr, ^{13}C nmr[65] and mass spectra[76] of the synthetic product were consistent with literature values.

METHYL-1-METHYL-1,2,3,4-TETRAHYDRO-β-CARBOLINE-3-CARBOXYLATE (**118**)

A sample of compound (**117**) (20 mg) was dissolved in the minimum amount of ethanol, to which diazomethane in ether was added. The resulting product was purified by flash column chromatography on C18 reverse phase material, using a water to methanol gradient, to yield 15 mg of (**118**) as a white powder, the mass spectrum of which was consistent with literature data[82].

NMR SPECTROSCOPY OF β-CARBOLINE ALKALOIDS

For ^{1}H and ^{13}C nmr spectral data of the natural product alkaloids and their derivatives, refer to Tables 2.1-2.2 and Table 2.4. For the ^{1}H and ^{13}C nmr spectral data of the synthetic alkaloids, refer to Tables 3.1-3.3.

ASSAY

Alkaloids were submitted for assay in the three biological assay systems outlined in Chapter One of this thesis, as methanol solutions. For the antimicrobial/antifungal assay, the MIC of the compounds against each test organism was determined from a series of two-fold dilutions.

PARTITION COEFFICIENT DETERMINATION

The method used for the determination of the partition coefficient of 1-propyl-β-carboline will be outlined as an example of the general method employed. The

experimentally determined partition coefficient is the average result of five such measurements.

PREPARATION OF EQUILIBRATED SOLVENTS

Distilled water and octanol were placed together in glass-stoppered 5 ml centrifuge tubes. The tubes were inverted 100 times over five minutes and then centrifuged for five minutes at 2000 rpm. The phases were transferred into separate flasks by means of droppers.

PREPARATION OF STANDARD SOLUTION

9.3 ± 0.05 ml methanol was added to a sample of 1-propyl-β-carboline (18.6 ± 0.0001 mg) with a 10 ml graduated pipette, to give a 2 ± 0.022 mg/ml solution.

DETERMINATION OF STANDARDS

0.25 ± 0.005 ml of the standard solution was transferred into each of two 10 ml volumetric flasks via a 1 ml graduated pipette and the solvent removed. The octanol standard was made up to the mark with equilibrated octanol, while the water standard was made up to the mark with equilibrated water and methanol (0.5 ml) to aid solubility. This yielded 10 ± 0.025 ml standard solutions. The uv spectra of each of the standards were recorded and the extinction coefficients of the compound in each solvent determined.

MEASUREMENT OF PARTITION COEFFICIENTS

1 ± 0.005 ml of the standard methanol solution of 1-propyl-β-carboline was transferred to a glass stoppered centrifuge tube via a 1 ml pipette and the solvent removed. 1 ± 0.005 ml each of equilibrated octanol and water were added, again via pipettes and the mixture shaken and centrifuged as for the solvent equilibration. Uv spectra of each phase were recorded, directly for the aqueous phase, and by dilution of the octanol phase. 0.25 ± 0.005 ml of this phase was withdrawn into a 10 ml volumetric flask and made up to a 10 ± 0.025 ml solution with equilibrated octanol. The concentration of compound in each phase was calculated from the absorption and the previously determined extinction coefficients and used to calculate log P. The average value of five such determinations was 2.50 ± 0.23 for 1-propyl-β-carboline (**113**) and 2.26 ± 0.21 for 1-isopropyl-β-carboline (**114**).

CALCULATION OF LOG P VALUES

a) Using substituent constants, π. The π value for a methylene group is 0.50[101]. Using the experimentally determined log P for 1-propyl-β-carboline (**113**) of 2.50, the values are calculated as follows. For 1-ethyl-β-carboline (**56**), log P = 2.50 - 0.50 = 2.00. The other log P values were calculated in a similar manner.

b) Using fragmental constants, *f*. The fragmental constant for a methylene group is 0.66, a hydrogen 0.23 and a methyl group 0.89[102]. Again using the value of 2.50 for log P of 1-propyl-β-carboline (**113**), log P of norharman (**58**) is calculated as 2.50 - (2 x 0.66) - 0.89 + 0.23 = 0.52.

DETERMINATION OF Rf VALUES

Silica gel plates (9.5 x 12.5 cm) were used. Alkaloids were spotted onto the plates as 1 mg/ml solutions in methanol. Plates were developed in a sealed glass chromatography chamber lined with filter paper which had been pre-soaked in the eluting solvent. Solvents systems used were either chloroform/methanol (9:1) or ethyl acetate/acetone (1:1). Plates were visualised under ultraviolet light and the compounds circled with pencil. The Rf values were calculated as the distance from the reference line to the middle of the compound spot, over the distance travelled by the solvent front. Four plates of each solvent system were developed and the results averaged, to yield a Rf value for each alkaloid in each solvent system. For Rf values of the alkaloids, refer to Table 3.8.

DETERMINATION OF HPLC RETENTION INDICES

Reverse phase hplc retention indices were determined using a Philips PU 4100 liquid chromatograph coupled to a Philips PU 4120 photo diode array detector. Alkaloids were injected as 1 mg/ml methanol solutions (5-10 µl per injection) onto a Hamilton analytical PRP-1 column, using methanol/water-TFA (3:1) and a flow rate of 1 ml per minute. The elution time for a non-retained compound (T_0) was determined to be 284 seconds from the retention time of methanol. Retention indices were measured as the time at which the maximum uv absorption was recorded for each compound. Multiple injections of each sample were run and the results averaged. For the hplc retention indices of the alkaloids, refer to Table 3.8.

WORK DESCRIBED IN CHAPTER FOUR

4.2 STUDIES OF THE SPONGE, *AXINELLA* SP. 2

COLLECTION AND EXTRACTION OF SPONGE

Axinella sp. 2, (200 g), was collected by SCUBA diving in April 1986 from the sponge garden, Goat Island, Leigh, off the North Island of New Zealand and stored frozen. A voucher specimen, 86L01-02, is held at the Chemistry Department, University of Canterbury.

Freshly thawed sponge, (196 g), was blended three times with methanol/toluene, (4:1), filtered and the solvent evaporated from the combined filtrates. The resulting extract (9.6 g), was chromatographed on a reverse phase C18 column (50 g), using a water/methanol to methanol/ dichloromethane gradient to give 12 fractions.

GIROLLINE (**153**)

Fractions 1, 2 and 3 from the reverse phase column showed significant activity on both P388 and antiviral/cytotoxicity assay systems. These fractions were combined (7.36 g), taken up in methanol/water (4:1) and filtered to leave 3.26 g of methanol soluble material, which was chromatographed on Fractogel PGM 2000 (400 g), using water as solvent. The activity was retained, but after continued elution with 0.25 M acetic acid (600 ml), crude girolline acetate (740 mg) was obtained, which was converted to the hydrochloride by addition of 10 ml conc. HCl, then taken to dryness. The sample was then dissolved in methanol and filtered to leave 477 mg of methanol soluble material. Semi-preparative high pressure liquid chromatography on a C18 RP Alltech column using methanol/water-TFA (0.05% TFA), 1:4, 4 ml/min. with uv detection at 220 nm, yielded a fraction of 297 mg of girolline, clean by ^{1}H nmr spectroscopy in D_2O. Elemental analysis of 5 mg of this fraction revealed only 1.5% carbon, indicating the presence of a large amount of inorganic salts. A further gel permeation column using 10 g of fractogel PGM 2000 and water as solvent, led to the isolation of a fraction of 24 mg of an opaque oil, again pure by nmr spectral analysis. ms FAB 367 $[M + matrix + Na]^{+}$, 331 $[M + matrix + Na - HCl]^{+}$; ^{1}H nmr (D_2O) 6.89 (1H, d, J=1.0 Hz, H-5), 5.15 (1H, d, J=3.5, 1.0 Hz, H-7), 4.59 (1H, dt, J=9.3, 3.5 Hz, H-8), 3.60 (1H, dd, J=13.8, 3.5 Hz, H-9), 3.44 (1H, dd, J=13.8, 9.3 Hz, H-10); ^{13}C nmr (CD_3OD) 149.15 (C-2), 128.06 (C-4), 112.07 (C-5), 67.97 (C-7), 62.22 (C-8), 44.52

(C-9); UV λ_{max} (MeOH) 213 nm; ir ν_{max} 3500 br, 3310, 2250, 1680, 1635, 1415, 610 cm^{-1}. For the isolation scheme used, refer to Figure A.3 in the Appendix.

TAURINE (**141**)

A fraction (1.3 g) from the first gel permeation column of the water soluble partition of the extract was shown to contain taurine by ^{1}H and ^{13}C nmr spectroscopy. On standing, a white precipitate settled out, the ^{1}H and ^{13}C nmr spectra of which were identical with those reported for taurine[143,144].

HOMARINE (**94**)

Fractions from the gel permeation columns of the water soluble material from the extract were analysed by ^{1}H nmr spectroscopy in D_2O. Homarine was identified in several fractions by comparison of the ^{13}C nmr data with literature values[78].

HYMENIALDISINE (**139**)

Over time, a pale yellow solid settled out of fraction 6 (584 mg) from the initial reverse phase flash column on the extract. The solvent was removed via a dropper to leave 220 mg wet solid. A small quantity of good quality crystals (10 mg), was obtained by recrystallisation from methanol but a better yield, (76 mg), was obtained by recrystallisation from methanol/water, (1:1) using TFA fumes to aid dissolution and TEA fumes to neutralise the solution to give pale yellow needles.
mp. 220° C (decomposition); ms m/z 323/325 found 322.9766, ($C_{11}H_{10}N_5O_2{}^{70}Br$ requires 322.9780); UV λ_{max} (MeOH) 346, 265, 228 nm (log ε = 4.13, 4.05, 4.05 respectively), λ_{max} (MeOH-KOH) 382, 275, 237 nm; ir ν_{max} 3320 br, 1720, 1630, 1490, 1370 cm^{-1}. For ^{1}H and ^{13}C nmr spectral data, refer to Tables 4.2 and 4.3.

X-RAY CRYSTALLOGRAPHIC MEASUREMENTS OF HYMENIALDISINE (**139**)

The X-ray crystallographic analysis was performed by Dr. Richard Coll, using a Nicolet XRD P3 four-circle diffractometer with $\lambda(MoK\alpha)$ = 0.71069 Å. Yellow crystals were obtained from a methanol solution. A specimen of dimensions 0.5 x 0.23 x 0.125 mm^3 was selected for the X-ray measurements. The cell parameters were determined by least squares refinement of 25 accurately centred reflections in the range $6 < 2\theta < 31°$.

Crystal data: $C_{11}H_{10}N_5O_2Br.CH_3OH$, monoclinic, a = 7.185 (3), b = 16.309 (6), c = 11.833 (4) Å, β = 92.90 (3)°, V = 1384.81 Å^3, D_{cal} = 1.71 g.cm^{-3}, space group $P2_{1/n}$, (z = 4), F(0 0 0) = 647.82.

Using ω scans at a scan rate of 250 scans per hour and a background scan ratio of 0.5, a total of 2839 reflections were collected over a scan range of 1.6 at a temperature of 143 K. Of these, 1234 reflections were unique and used in the structure refinement. Crystal stability was monitored by recording three check reflections every 100 reflections and no significant variations were observed. The data was corrected for Lorentz polarisation effects. The structure was solved by the Patterson heavy-atom method. Hydrogen atoms were inserted at calculated positions using a riding model and the thermal parameters refined from a starting value of 0.05. The refinement using least squares procedures converged with R = 0.0538, ωR = 0.0394 and a maximum least-squares shift error of 0.006. The function minimised in the refinement was $\Sigma\, \omega(|F_o| - |F_c|)^2$ where $\omega = [\sigma^2(F_o) + 0.00121\, F_o^2]^{-1}$. All programs used for the data collection and structure solution are contained in the SHELXTL (Version 4.0) package[222].

Table 8.1: Bond angles (°) for hymenialdisine

C(2) - N(1) - C(5)	108.4(6)	C(14) - N(13) - C(12)	107.0 (7)
C(14) - N(15) - C(11)	108.5 (6)	N(13) - C(14) - N(15)	112.7 (6)
N(13) - C(14) - N(14)	125.4 (7)	N(15) - C(14) - N(14)	121.9 (7)
O(12) - C(12) - N(13)	126.5 (8)	O(12) - C(12) - C(11)	125.1 (7)
N(13) - C(12) - C(11)	108.4 (7)	Br - C(2) - N(1)	120.4 (6)
Br - C(2) - C(3)	128.7 (6)	N(1) - C(2) - C(3)	110.8 (7)
C(10) - C(4) - C(5)	124.6 (6)	C(10) - C(4) - C(3)	128.6 (6)
C(5) - C(4) - C(3)	106.0 (7)	C(4) - C(10) - C(11)	120.4 (7)
N(1) - C(5) - C(4)	108.6 (7)	N(1) - C(5) - C(6)	119.1 (7)
C(4) - C(5) - C(6)	131.6 (7)	N(15) - C(11) - C(12)	103.4 (6)
N(15) - C(11) - C(10)	125.9 (7)	C(12) - C(11) - C(10)	130.5 (7)
O(6) - C(6) - N(7)	122.1 (7)	O(6) - C(6) - C(5)	121.3 (7)
N(7) - C(6) - C(5)	116.6 (7)	C(2) - C(3) - C(4)	106.1 (7)
O_m - C_m - H_m(1)	109.0 (4)	O_m - C_m - H_m(2)	109.8 (4)
H_m(1) - C_m - H_m(2)	109.5	O_m - C_m - H_m(3)	109.6 (4)
H_m(1) - C_m - H_m(3)	109.5	H_m(2) - C_m - H_m(3)	109.5

Table 8.2 : Atomic coordinates (x 10) and temperature factors (Å x 10) for hymenialdisine.

Atom	x	y	z	U
Br	985 (1)	1496 (1)	328 (1)	25 (1)*
O(12)	5789 (7)	5712 (3)	-2344 (4)	16 (1)
O(6)	1403 (8)	2276 (3)	-4194 (4)	26 (2)*
N(7)	978 (8)	3662 (4)	-4139 (5)	17 (2)*
N(1)	1011 (9)	2206 (4)	-1833 (5)	16 (2)*
N(13)	7687 (10)	507 (4)	-957 (5)	15 (2)*
N(15)	5819 (9)	4005 (3)	-573 (5)	18 (2)*
C(14)	7463 (11)	4384 (4)	-328 (7)	14 (3)*
N(14)	8675 (9)	4101 (3)	456 (5)	16 (2)*
C(12)	6114 (12)	5158 (5)	-1638 (7)	16 (3)*
C(8)	603 (11)	4419 (4)	-3504 (6)	17 (3)*
C(2)	1454 (12)	2320 (5)	-720 (7)	19 (3)*
C(4)	2259 (10)	3478 (6)	-1635 (6)	13 (3)*
C(10)	3191 (11)	4238 (4)	-1944 (6)	12 (3)*
C(5)	1454 (11)	2919 (4)	-2410 (7)	13 (2)
C(11)	4848 (11)	4454 (4)	-1421 (6)	14 (3)*
C(6)	1284 (11)	2924 (5)	-3636 (7)	16 (3)*
C(3)	2267 (10)	3075 (4)	-533 (6)	15 (3)*
C(9)	2338 (11)	4782 (5)	-2898 (7)	19 (3)*
O_m	-1252 (8)	6264 (3)	-3427 (4)	26 (2)*
C_m	-1007 (13)	7037 (5)	-2889 (7)	30 (4)*
$H_m(1)$	-2054	7380	-3098	52 (8)
$H_m(2)$	115	7290	-3126	52 (8)
$H_m(3)$	-927	6965	-2083	52 (8)

* Equivalent isotropic U defined as one third of the trace of the orthogonalised U tensor.

Table 8.3 : Bond lengths (Å) for hymenialdisine (**139**)

Br - C(2)	1.870 (8)	O(12) - C(12)	1.244 (9)
O(6) - C(6)	1.252 (9)	N(7) - C(6)	1.356 (10)
N(1) - C(2)	1.352 (10)	N(1) - C(5)	1.393 (10)
N(13) - C(14)	1.360 (10)	N(13) - C(12)	1.362 (11)
N(15) - C(14)	1.352 (10)	N(15) - C(11)	1.404 (9)
C(14) - N(14)	1.322 (10)	C(12) - C(11)	1.500 (11)
C(2) - C(3)	1.377 (10)	C(4) - C(10)	1.464 (11)
C(4) - C(5)	1.398 (11)	C(4) - C(3)	1.460 (10)
C(10) - C(11)	1.353 (11)	C(5) - C(6)	1.449 (11)
O_m - C_m	1.419 (9)	C_m - H_m(1)	0.960
C_m - H_m(2)	0.960	C_m - H_m(3)	0.960

OROIDIN

After time, fractions 4 to 7 from the initial reverse phase flash column on the extract, which had been shown to contain oroidin by tlc and ^{1}H and ^{13}C nmr spectroscopy were reexamined by ^{1}H and ^{13}C nmr spectroscopy in DMSO-d6. No signals characteristic of oroidin were observed. A sample of 103 g of a fresh collection of *Axinella* sp. 2 (88L02-01) (96 g) was extracted in the manner described above, to yield 5.2 g of crude extract. The resulting extract was chromatographed on a reverse phase flash column using C18 material (50 g) and the solvent gradient employed in the first extraction, to yield 12 fractions. Fraction 7 from this column was shown to contain oroidin by ^{1}H and ^{13}C nmr spectroscopy. Various combinations of mplc and hplc were attempted on this fraction without success. Semi-preparative hplc using a cyanopropyl column, methanol/water-TFA (2:3), a flow rate of 5 ml per minute and uv detection at 280 nm, resulted in the isolation of a fraction (3 mg), the tlc and spectral properties of which were consistent with those of oroidin[116,134].

TLC SURVEY OF AXINELLIDA

Samples surveyed were as listed in Table 4.4. Survey samples were each 2 g which had previously been extracted using methanol/toluene (3:1), made up to a volume of 20 ml and the solution stored at -18°C. Samples were spotted at a high loading on silica gel tlc plates, which were developed using ethyl acetate/acetone/formic acid/water (10:6:1:1). Plates were visualised under ultraviolet

light and by dipping in Sakaguchi reagent[145]. Pure hymenialdisine and oroidin and a sample of crude *Axinella* sp. 2 extract (86L01-02) were used as references. A sub-sample was taken of an extract thought to contain hymenialdisine, namely 87MS02-04, and this sample was screened by the procedure outlined in Chapter Seven of this thesis. For the results of this screening, also refer to Chapter Seven, p. 144.

ISOLATION OF STEROLS

Fraction ten from the initial reverse phase flash column on the second extraction of sponge was examined by ^{1}H and ^{13}C nmr spectroscopy in $CDCl_3$, which indicated that it consisted mainly of sterols. A sub-sample of this fraction (40 mg), was subjected to normal phase column chromatography on silica gel (10 g), using a petroleum ether to ethyl acetate gradient. Fractions shown to contain sterols by tlc (dichloromethane/methanol (9:1), by visualisation with anisaldehyde dip), were combined. This combined fraction (30 mg), was shown to consist mainly of (**154**) by comparison of the ^{13}C nmr data with literature values[146].

A sample of 25 mg from this combined fraction was despatched to Professor Carl Djerassi for gcms analysis of the sterol content.

WORK DESCRIBED IN CHAPTER FIVE

5.1 STUDIES OF THE SPONGE, *STYLOPUS AUSTRALIS*

COLLECTION AND EXTRACTION OF SPONGE

Stylopus australis n. sp. (185 g wet) was collected by SCUBA diving in April 1986 from Goat Island Canyon, Leigh, off the North Island of New Zealand. A voucher specimen, 86L02-01, is held at the Chemistry Department, University of Canterbury. The sponge was stored frozen, and then in a typical extraction, 85 g was blended with methanol/toluene (4:1). The resulting extract (4.4 g) was partitioned between ethyl acetate and water. The extract from the aqueous phase (3.8 g) was taken up in methanol, and filtered to remove an insoluble purple pigment.

3β,4β-DIHYDROXY-PREGN-5-EN-20-ONE-3-SULPHATE (**164**)

The methanol soluble extract (3.2 g) was chromatographed on a reverse phase C18 column (50 g), using a water/methanol to methanol/dichloromethane gradient[223]. Fraction 5 (182 mg) was rechromatographed on a column of silica gel (5

g), using a dichloromethane/methanol gradient, yielding fractions 7-12 which were pure by tlc (silica gel, dichloromethane/methanol (3:1), blue-purple spot with vanillin/H_2SO_4 reagent) and by ^{13}C nmr spectroscopy. These were recombined to give a white solid (101 mg), recrystallised from ethyl acetate/methanol as white needles.

mp 120-121°C; $[\alpha]_D^{25}$ = -100°, (c = 0.1, MeOH); cd (MeOH) $\Delta\varepsilon$ (nm) 0 (324), +11 (284), 0 (240); ord (MeOH) θ (nm) +4.5 (302), 0 (286), -9.2 (257); ms m/z found 411.1833 $[M^+ -H]^-$ ($C_{21}H_{31}O_6S$ requires 411.1841), 331 $[M^+-HSO_3]^-$, 313 $[M^+-H-H_2SO_4]^-$; ir υ_{max} (KBr) 3600 br, 2995, 1700, 1620, 1460, 1385, 1360, 1255, 1215 cm^{-1}. For ^1H and ^{13}C nmr spectral data, refer to Tables 5.1 and 5.2.

3β,4β-DIHYROXY-PREGN-5-EN-20-ONE-3-SULPHATE-4-ACETATE (**173**)

Sulphate (**164**) (10 mg) was treated with acetic anhydride (2 ml) and pyridine (2 ml) at room temperature overnight. Water (1 ml) and one drop of HCl (2 M) were added, and the product (**173**) extracted with chloroform (3 x 0.5 ml) as a brown oil. This was recrystallised from chloroform to yield (**173**) (8 mg) as a white solid.

mp 122-123°C; ms m/z 453 $[M-H]^-$, 395 $[M-C_2H_3O_2]^-$; ir ν_{max} (smear) 2995, 1735, 1705, 1635, 1460, 1380, 1235, 1195 cm^{-1}. For ^1H and ^{13}C nmr spectral data, refer to Tables 5.1 and 5.2.

3β,4β,20β-TRIHYDROXY-PREGN-5-ENE-3-SULPHATE (**174**)

Sulphate (**164**) (5 mg) was dissolved in ethanol (0.5 ml) and excess $NaBH_4$ added. The reaction was left stirring and monitored by tlc. After two hours the reaction mixture yielded a single spot on tlc at Rf = 0.45 (silica gel, dichloromethane/methanol (3:1), purple spot with vanillin/H_2SO_4 reagent). The crude product was purified by preparative tlc on silica gel (6 x 8 cm), using the same conditions as above, but dichloromethane/methanol (1:1) was used to initially move the compound off the baseline. Visualisation of a small strip of silica with vanillin/H_2SO_4 reagent led to the location of the product, which was eluted with methanol and filtered to yield 3 mg of product (**174**), pure by ^1H and ^{13}C nmr spectroscopy.

ord (MeOH) θ (nm) -8.5 (280); ms FAB found 459.1794 $[M-H+2Na]^-$ ($C_{21}H_{33}O_6SNa_2$ requires 459.1794), 414 $[M]^-$; ir ν_{max} (smear) 3560 br, 2950, 2900, 1580, 1460, 1430,

1380, 1240, 1090 cm^{-1}. For ^{1}H and ^{13}C nmr spectral data, refer to Tables 5.1 and 5.2.

ACID HYDROLYSIS OF SULPHATE (**164**) IN HCl[224]

5 mg of (**164**) was dissolved in methanol (0.5 ml)/2 M HCl (1 ml). The closed vial was put in a water bath over steam for three hours, with occassional shaking. After cooling, the sample was extracted with chloroform (3 x 1 ml) to give 3.4 mg of a dark brown oil. Tlc analysis showed no clearly defined spots and a ^{1}H nmr spectrum revealed no identifiable signals.

BASE HYDROLYSIS OF SULPHATE (**164**)[225]

7 mg of (**164**) was dissolved in pyridine/dioxane (0.5 ml of each) and heated in an oil bath at 110°C under a nitrogen atmosphere with monitoring by tlc. After 15 minutes, the solution had darkened in colour and tlc showed no starting material was present. The sample was partitioned between ethyl acetate and water and a ^{1}H nmr spectrum obtained of each fraction, with no clearly identifiable signals.

BASE HYDROLYSIS OF SULPHATE (**174**)

4 mg of (**174**) was dissolved in pyridine/dioxane (0.5 ml of each), heated in an oil bath at 110°C and the reaction monitored by tlc. After 72 hours, both tlc and ^{1}H nmr spectroscopy indicated that no reaction had occurred.

MILD ACID HYDROLYSIS OF SULPHATE (**164**)[225]

5 mg of (**164**) was dissolved in 50% acetic acid (1 ml) and solid barium hydroxide added to bring the pH to 4.5. 0.1 ml of methanol was added to aid solubility and the mixture heated to 60°C in an oil bath. After 72 hours both tlc and ^{1}H nmr spectroscopy indicated that no reaction had occurred and addition of more acetic acid failed to produce a result. The same reaction was repeated without the buffer present, but again no reaction occurred.

REDUCTION OF SULPHATE (**164**) BY LITHIUM ALUMINIUM HYDRIDE

3 mg of (**164**) and a ten fold excess of lithium aluminium hydride (3 mg) were dissolved in 1 ml THF previously dried over "LithAl" and heated with stirring at 70°C in an oil bath. The reaction was monitored by tlc which indicated at least 3 products,

none of which gave a positive sulphate test with methylene blue dip[163]. The ^1H nmr spectrum of the reaction mixture in $CDCl_3$ showed no recognisable signals.

BIOLOGICAL ACTIVITY OF SPONGE

The organic phase of the sponge from the initial partition (432 mg) was chromatographed on a silica gel column (15 g), using a dichloromethane/methanol gradient to yield 9 fractions. Fraction 4 from this column (71 mg) was rechromatographed on a silica gel column (1.7 g), using an ethyl acetate/ethanol gradient to yield 14 fractions, of which fractions 8-11 showed biological activity in the antiviral/cytotoxicity assay system. After obtaining the ^1H nmr spectrum of each of these fractions in $CDCl_3$, further work was abandoned as it was apparent that they still contained complex mixtures of products and there were few similarities between the fractions.

2,3-DIHYDROXY-1-HEXADECYLOXYPROPANE (CHIMYL ALCOHOL) (**175**)

The organic phase from the ethyl acetate/water partitioning of the sponge extract (432 mg) was chromatographed on silica gel (15 g), using a dichloromethane/methanol gradient. Fraction 4 was rechromatographed on silica gel (1.7 g), using an ethyl acetate/ethanol gradient to yield 5 mg of a yellow oil, the ^1H and ^{13}C nmr and mass spectra of which, corresponded to the literature data for the chimyl alcohol[165,90].

IDENTIFICATION OF STEROLS

The ^1H and ^{13}C nmr spectra of a fraction from a silica column of the extract were used to identify cholesterol and 24-methylene cholesterol by comparison with the literature data[81,226]. These identifications were confirmed by subsequent gcms of the TMS ethers of the sterols. The minor sterols were also identified by gcms of the TMS ethers.

TLC SURVEY OF HYMEDESMIIDAE SPONGES

Sponges surveyed were *Stylopus* sp. 1 (87L04-3), *Stylopus australis* (86L02-01), *Stylopus* sp. (87K14-01), *Hymedesmia* sp. 1 (87WH01-04), *Hymedesmia* sp. 2 (87K01-01) and three *Hymedesmia* species (84L03-27), (84L02-28) and (84K07-13) respectively. The survey samples were each 2 g which had

previously been extracted using methanol/toluene (3:1), made up to a volume of 20 ml and the solution stored at -18°C. Samples were spotted at high loading on a silica tlc plate which was developed using dichloromethane/methanol (3:1) as eluting solvent. Pure (**164**) was used as a reference. Samples were visualised with vanillin/H_2SO_4 reagent. Any samples appearing to contain (**164**) were extracted on a small scale (10 g), using methanol/toluene (3:1) and a portion of the extract submitted to flash column chromatography using C18 reverse phase material. The appropriate fractions were checked for the presence of (**164**) by ^1H nmr spectroscopy in CD_3OD.

ISOLATION OF STEROL MIXTURES

20 g of each sponge was extracted by blending with chloroform (3 x 50 ml). Solvent was then removed under reduced pressure and the sample redissolved in chloroform. The solvent was again removed to typically yield 30-50 mg of extract. This extract was chromatographed on a column of silica gel (2 g), using a petroleum ether/ethyl acetate gradient. Fractions containing sterols by tlc comparison with cholesterol, (petroleum ether/ethyl acetate (4:1), purple spot with vanillin/H_2SO_4 reagent), were combined and used for the preparation of TMS ethers.

PREPARATION OF TMS ETHERS[147]

In a typical derivatisation, sterol mixtures were dissolved in ethyl acetate, transferred to a centrifuge tube and dried under nitrogen. A stirrer was added and the tube sealed with a rubber septum. Pyridine (500 µl), hexamethyldisilazane (200 µl) and trimethylsilylchloride (TMSCl), (100 µl), were added, and the solution left stirring under a nitrogen atmosphere for thirty minutes. After cooling in ice water, water (0.5 ml) and hexane (0.5 ml) were added. The organic layer was transferred by pipette to a conical flask and the aqueous layer extracted twice more with hexane. The combined organic extracts were dried over Na_2SO_4 and filtered. The solvent was removed under a nitrogen atmosphere and again the tube was sealed with a rubber septum.

GCMS ANALYSES

The sample was dissolved in the minimum amount of petroleum ether and 2 µl of this solution was injected onto the gcms capillary column. General retention

time range was between 80-110 minutes. For each peak for each sample, a mass spectrum was obtained as a sum of best scans with background subtraction, and the sterols present identified by comparison of relative retention times and mass spectral fragmentation patterns with literature data. Percentage composition was determined by gas chromatography.

5.2 STUDIES OF THE SPONGE, *HYMEDESMIA* SP. 1

COLLECTION AND EXTRACTION OF SPONGE

Hymedesmia sp. 1 (400 g wet) was collected by SCUBA diving in July 1987 from Wellington Harbour off the North Island of New Zealand. A voucher specimen, 87WH01-04, is held at the Chemistry Department, University of Canterbury. The orange sponge was stored frozen, then 112 g was blended with methanol/toluene (4:1). The resulting extract (6.6 g) was partitioned between chloroform and water.

D-RHODOIC ACID (**182**)

The extract from the aqueous phase (5.4 g) was chromatographed on a reverse phase C18 flash column (80 g), using a water to methanol gradient to yield 10 fractions. A ^{1}H nmr spectrum of the first fraction from this column in D_2O, revealed the presence of several components, present in sufficient quantity to warrant further investigation. This fraction (4.4 g) was further partitioned by gel permeation chromatography on Fractogel PGM 2000 (400 g), in water. ^{1}H nmr spectroscopy in D_2O revealed that the major component was concentrated in five fractions from this column which eluted just before those containing salt. These were recombined (572 mg) and subjected to further gel permeation chromatography, using water as solvent. ^{1}H nmr spectra in D_2O were obtained of fractions taken at various intervals from the column. Some of those fractions containing desired components were subjected to reverse phase hplc on an Alltech C8 semi-preparative column, using methanol/water (2:3) as solvent, with a flow rate of 4 ml per minute and uv detection at 220 nm. This led to the isolation of rhodoic acid (**182**) (80 mg) as a clear oil.

ms SIMS (TEA) 234 $[M-3H+K]^-$, 218 $[M-2H+Na]^-$, 196 $[M-H]^-$; ^{1}H nmr (D_2O) 3.23 (2H, t, J=6.8 Hz, H-1), 3.42 (2H, t, J=6.8 Hz, H-2), 4.00 (1H, q, J=7.1 Hz, H-3), 1.51 (3H, d, J=7.1 Hz, H-4); ^{13}C nmr (D_2O) 47.40 (C-1), 42.34 (C-2), 57.25 (C-3), 14.82 (C-4), 173.16 (C-5). Ir, cd and ord data were in accord with literature values[174].

HOMARINE (**94**)

Fractions from the initial gel permeation column which eluted later than those containing D-rhodoic acid, contained salt and one other major organic component. This component was identified as homarine from comparison of the ^{13}C nmr data with literature values[78].

TAURINE (**141**)

A fraction from the hplc purification of D-rhodoic acid contained a single component which gave a pink spot with ninhydrin spray on tlc. This component was identified as taurine by comparison of its ^{1}H and ^{13}C nmr spectra with those previously reported for taurine[143,144].

BIOLOGICAL ACTIVITY OF SPONGE

The organic phase (1.1 g) of the initial chloroform/water partition of the crude sponge extract, was further partitioned by normal phase flash column chromatography on silica gel (40 g), using a petroleum ether to ethyl actetate gradient, to yield 18 fractions. The biological activity was located in the last six fractions from the column by antiviral/cytotoxicity assay, so these were recombined (245 mg) and rechromatographed on a C18 reverse phase flash column (5 g), using a water to methanol to dichloromethane gradient to yield 13 fractions. Antiviral/cytotoxicity assay showed the biological activity to be localised in fractions 5-9. The most active of these was analysed by reverse phase hplc on an Alltech C18 analytical column, using methanol/water (7:3), a flow rate of 1 ml per minute and uv detection at 210 nm. Six fractions were collected and analysis by the antiviral/cytotoxicity assay indicated that the biological activity was spread throughout the column. Hplc on the other combined active fractions produced the same result. A ^{1}H nmr spectrum in CD_3OD of the first fraction from the hplc column, contained no clearly recognisable features, comprising very broad unresolved humps throughout the spectral range.

A re-extraction of 103 g of sponge was performed as outlined previously, to yield 5.9 g of crude sponge extract. Partitioning of a sub-sample of the extract (58 mg) between chloroform and water did not result in the activity partitioning cleanly, so the entire crude extract was subjected to reverse phase flash column chromatography on C18 material (80 g), using a water to methanol to

dichloromethane gradient as previously, to yield 11 fractions. The biological activity was concentrated into fractions 4-7 and reverse phase hplc on the most active fraction by antiviral/cytotoxicity assay (fraction 5), again resulted in activity being spread throughout the column fractions.

Gel permeation was attempted using a fraction (30 mg) from a reverse phase column of the first extraction of the sponge. This was chromatographed on Fractogel PGM 2000 (40 g), in methanol to yield 20 fractions. Activity was spread through the fractions, but was most concentrated in the latter five fractions from the column, indicating perhaps that the active component is a small molecule.

As both tlc and ^{1}H nmr spectroscopy of fractions from the column were uninformative and no suitable means of concentration of the activity had been found, work on the biological activity of the sponge was abandoned.

WORK DESCRIBED IN CHAPTER SIX

6.1 STUDIES OF THE SPONGE, *HYMENIACIDON HAURAKI*

COLLECTION AND EXTRACTION OF SPONGE

Hymeniacidon hauraki was collected by SCUBA diving in February 1987 from Takatu Point and Leigh Reef, Leigh, off the North Island of New Zealand. The orange sponge was stored frozen and then 194 g of the Takatu Point sample was blended with methanol/toluene (4:1), filtered under vacuum and the solvent removed to yield 8.5 g of crude extract.

ISOLATION OF FURAN FATTY ACID (**183**)

510 g of *H. hauraki* was blended as outlined previously to yield 16.6 g of crude extract. This was partitioned between chloroform and water and the organic phase (410 mg) subjected to column chromatography on silica gel (15 g), using a petroleum ether to ethyl acetate gradient to yield 10 fractions. Fraction 7 from this column (35 mg), was used for nmr spectroscopic work, including ^{1}H, ^{13}C, HETCOR, COSY and DEPT experiments. Fractions 7-9, shown to contain (**183**) by tlc and ^{1}H nmr spectroscopic analysis were recombined (71 mg), for further chromatographic purification on silica gel (2 g), using a petroleum ether to ethyl acetate to ethanol gradient to yield 10 fractions. Combination of fractions 2 and 3 from the column gave

37 mg of (8*Z*, 11*Z*, 14*Z*, 17*Z*)-3,6-epoxyeicos-3,5,8,11,14,17-hexenoic acid (**183**) as a yellow oil. For the isolation scheme used, refer to Figure A.5 in the Appendix. ir (smear) ν_{max} 3420, 2960, 1720, 1575, 1410, 1230, 1020, 990, 790, 720 cm^{-1}; UV λ_{max} (MeOH) 220, 281 nm (log ε = 3.13, 2.40 respectively); HETCOR, H2 <-> $C_{33.80}$, H4 <-> $C_{108.99}$, H5 <-> $C_{105.99}$, H7 <-> $C_{26.28}$, H8 <-> $C_{130.07}$, H9 <-> $C_{124.64}$, H10,13,16 <-> $C_{25.59}$. For ^1H and ^{13}C nmr spectral data, refer to Table 6.1.

ATTEMPTED METHYLATION OF FURAN FATTY ACID (**183**)

A sub-sample of (**183**) (2 mg) was dissolved in chloroform and diazomethane in ether added. Tlc analysis of the reaction mixture versus starting material (petroleum ether/ethyl acetate (3:1), visualisation with iodine vapour), revealed the presence of two spots at Rf values of 0.81 and 0.87 for the reaction mixture and two spots at Rf values of 0.17 and 0.26 in the starting material. ^1H nmr spectra of each sample in $CDCl_3$ indicated that decomposition was occurring and this was complete before further characterisation could be achieved.

RE- EXTRACTION OF SPONGE

50 g of *H. hauraki* (87TP01-09) was extracted in the manner previously described, except that dichloromethane was used instead of toluene, the blender was flushed with nitrogen and β-carotene (200 mg) was added to the extract prior to blending. The resultant extract (2.44 g) was dry-coated to celite (1 g) and partitioned by flash column chromatography on C18 reverse phase material using a water to methanol to dichloromethane gradient to yield 11 fractions. Fractions 5 and 6 from this column were shown to contain the furan compound by both tlc (petroleum ether/ethyl acetate (1:4), strong spot at Rf = 0.5, visualisation with iodine vapour) and ^1H nmr analysis. These fractions were recombined (47 mg) for further chromatography. The ethyl acetate soluble material from these fractions (21 mg) was injected onto a diol lobar column which was run with petroleum ether/ethylacetate (4:1) as solvent, 5 ml per minute with both refractive index (RI) and uv detection. A total of 60 20 ml fractions was collected. Fractions were evaporated down and those in which no mass was detected were combined in groups. Examination of the fractions by tlc indicated that the furan compound was in the combined fractions 16-21 (1 mg) and combined fractions 22-27 (0.5 mg) as a yellow oil, pure by ^1H nmr spectroscopy.

PREPARATION OF FURAN FATTY ACID METHYL ESTER (**184**)

As attempts to obtain mass spectra of the furan fatty acid were unsuccessful, the methyl ester was prepared. A sub-sample of the furan fatty acid (250 μg) was dissolved in methanol, diazomethane in ether was added and the solution left for three hours. After this time, tlc analysis (petroleum ether/ethyl acetate (1:1), visualisation with iodine vapour) revealed that the reaction mixture contained a single product at Rf = 0.9. This product was used for mass spectral analysis.
ms m/z found $M^{+\cdot}$ 328.2033, ($C_{21}H_{28}O_3$ requires $M^{+\cdot}$ 328.2038).

ISOLATION OF CORBISTEROL (**186**) AND THE PEROXIDE (**187**)

The extract was chromatographed on a reverse phase C18 column (50 g) using a water/methanol to methanol/dichloromethane gradient to yield 13 fractions. Fractions 8 and 9 from this column were recombined (90 mg) and a sub-sample (35 mg) was further partitioned by column chromatography on silica gel (0.8 g), using a petroleum ether to ethyl acetate gradient to yield 8 fractions. Fraction 4 from this column (0.8 mg) contained a major component, the ^{1}H, ^{13}C nmr and mass spectra of which, were used to identify it as corbisterol (**186**) by comparison with literature data. This identification was confirmed by subsequent gcms analysis of the TMS ethers of the sterol mixture of the sponge.

Fraction 5 from the column (2 mg), contained the peroxide (**187**) which was identified by comparison of the spectroscopic data with literature values[202].

ISOLATION OF STEROL MIXTURES

20 g of each sponge sample (87TP01-09) and (87LR01-05) was extracted with chloroform and the sterol mixture purified by an identical method to that used for the sterol mixtures analysed in Chapter Five of this thesis. Preparation of TMS ethers of the sterols and subsequent gcms analyses were also carried out by procedures identical to those outlined previously, (see Work Described in Chapter Five, p. 175).

SCREENING OF INDIVIDUAL SPONGES

Several samples of *H. hauraki* were divided into individual sponges and bagged separately. Samples were 87TP01-09 (six individuals), 88L01-01 (four individuals), 87SI01-09 (four individuals), 87L02-47 (one individual) and 88TAB02-02 (one individual). 20 g of each individual was extracted with chloroform,

filtered and the solvent removed. ^{1}H and ^{13}C nmr spectra of each extract were obtained in $CDCl_3$. For each sample, $\Delta^{5,7}$ sterols were detected, and in five cases, peroxide formation was evident also.

WORK DESCRIBED IN CHAPTER SEVEN

7.1 "FINGERPRINTING" OF MARINE NATURAL PRODUCT EXTRACTS

EXTRACTION OF ORGANISM

In a typical extraction, 10 g of the organism was blended in methanol/toluene (3:1), (3 x 50 ml), using a Janke and Kunkel, Ika-Werk blender. The extract was filtered by vacuum and the solvent removed to yield approximately 0.5 g extract.

PREPARATION OF EXTRACT

For each column, approximately 10 mg of celite, pre-washed with methanol, was added to approximately 20 mg of crude extract in solution and the mixture was evaporated to dryness on a rotary evaporator. The celite was then scraped off the inside of the flask and carefully loaded onto the prepared column.

PREPARATION OF COLUMN

Pasteur pipettes containing 200 mg of the appropriate stationary phase, were used for chromatography. The stationary phase was slurry packed into the column in methanol for the C18 reverse phase and phenyl reverse phase columns and equilibrated back to water, before introduction of the extract. The silica gel was slurry packed in petroleum ether.

CHROMATOGRAPHY

Fractions were 1.5 ml, (approximately 3 column volumes), in each case. Pressure was applied to the column by use of a rubber eye dropper bulb. Solvent composition was as follows: for both C18 reverse phase and phenyl reverse phase chromatography, fraction 1 was distilled water, fraction 2, water/methanol (1:1), and fraction 3, methanol. For the silica gel chromatography, fraction 1 was petroleum ether, fraction 2, dichloromethane, fraction 3, ethyl acetate and fraction 4, ethanol.

ASSAY

The crude extract was assayed for biological activity in antimicrobial, antiviral/cytotoxicity and P388 assay systems. The extract was re-assayed a week later to check the stability of the biological activity, and then the activity was diluted out until a negative assay reading was obtained on the antiviral/cytotoxicity assay system. All fractions were initially submitted for antiviral assay at 1 mg/ml, 20 µl per cell well. Those fractions which showed activity at this level were diluted ten fold until a negative assay reading was obtained. In this way the minimum inhibitory concentration was determined. The fractions were also submitted for P388 assay at appropriate concentrations, as determined by the antiviral assay results, to obtain an IC_{50} value in ng/ml for each fraction.

ION EXCHANGE CHROMATOGRAPHY

Columns used were commercial Alltech Extract-elute cartridges, each containing 100 mg of either SAX or SCX ion exchange resin. Each sample to be chromatographed was dry coated to celite (20 mg) and scraped into a small vial. 0.025 M ammonium acetate (0.5 ml) was added and the sample divided in two for SAX and SCX ion-exchange chromatography.

Each cartridge was prepared by eluting with methanol (1 ml), then 0.025 M ammonium actetate (1 ml). The sample slurry was loaded onto each column with a dropper. Fractions were 1.5 ml in each case and solvent was passed slowly through the column without pressure. Solvent composition was as follows: for SAX; fraction 1 was ammonium acetate (0.025 M), fraction 2, acetic acid (0.5 M) and fraction 3, sodium citrate (0.5 M). For SCX, fraction 1 was again ammonium acetate (0.025 M) and fraction 2, acetic acid (0.5 M).

Samples put through this process were fractions from the chromatography columns on extracts of *Axinella* sp. 2, *Ritterella sigillinoides* and *Latrunculia brevis*. For each extract, an active column fraction of an appropriate mass was selected.

The ion exchange column fractions were dried and submitted for assay in the antiviral/cytotoxicity assay system in methanol (100 µl).

GEL PERMEATION CHROMATOGRAPHY

The column used was a hplc analytical 5 µ PL gel column with 50 Å pore size from Polymer Laboratories. Solvent used was acetonitrile or acetonitrile with 0.5%

glacial acetic acid added and flow rate was 1 ml per minute with uv detection at 200 nm. The column was calibrated with acetonitrile solutions of pure compounds (toluene, polyethylene glycol and mycalamide A (**210**) and with a mixture of these compounds. The column volume was determined to be 8 ml.

Active column fractions from the initial chromatography columns on the extracts and pure active compounds were injected as approximately 1-3 mg/ml acetonitrile solutions. For each sample, eight fractions were collected, equivalent to a total of three column volumes of solvent. Fractions were dried down and submitted for P388 assay in methanol (200 µl).

The column was recalibrated in methanol using toluene, napthalene, 1,4-dibromonapthalene, bromothymol purple, bromocresol purple, bromocresol green and disulphine blue, with uv detection at an appropriate wavelength. Elution times of these compounds were inconsistent with the molecular weights and with the results obtained in acetonitrile.

APPENDIX:

SEPARATION TREES

KEY TO USE OF SEPARATION TREES.

ABBREVIATIONS

RP column	reverse phase (C18) flash chromatography column.
Davisil column	silica column chromatography.
Fractogel PGM 2000	Gel permeation column.
pTLC	preparative thin layer chromatography.
AM	antimicrobial assay.

FORMAT

A typical example is shown below.

MP5 47.5

24 mg

92

ww ww 1+ C7*

girolline (**153**)

MP5 47.5 refers to the code number - Michèle Prinsep, book 5, page 47, sample 5.

24 mg is the sample mass.

92 is the IC_{50} value for the sample (ng/ml).

ww ww 1+ C7* is the antiviral/cytotoxicity assay result, expressed as in Section 1.2 of this thesis, p. 7.

girolline (**153**) refers to the compound of this text number.

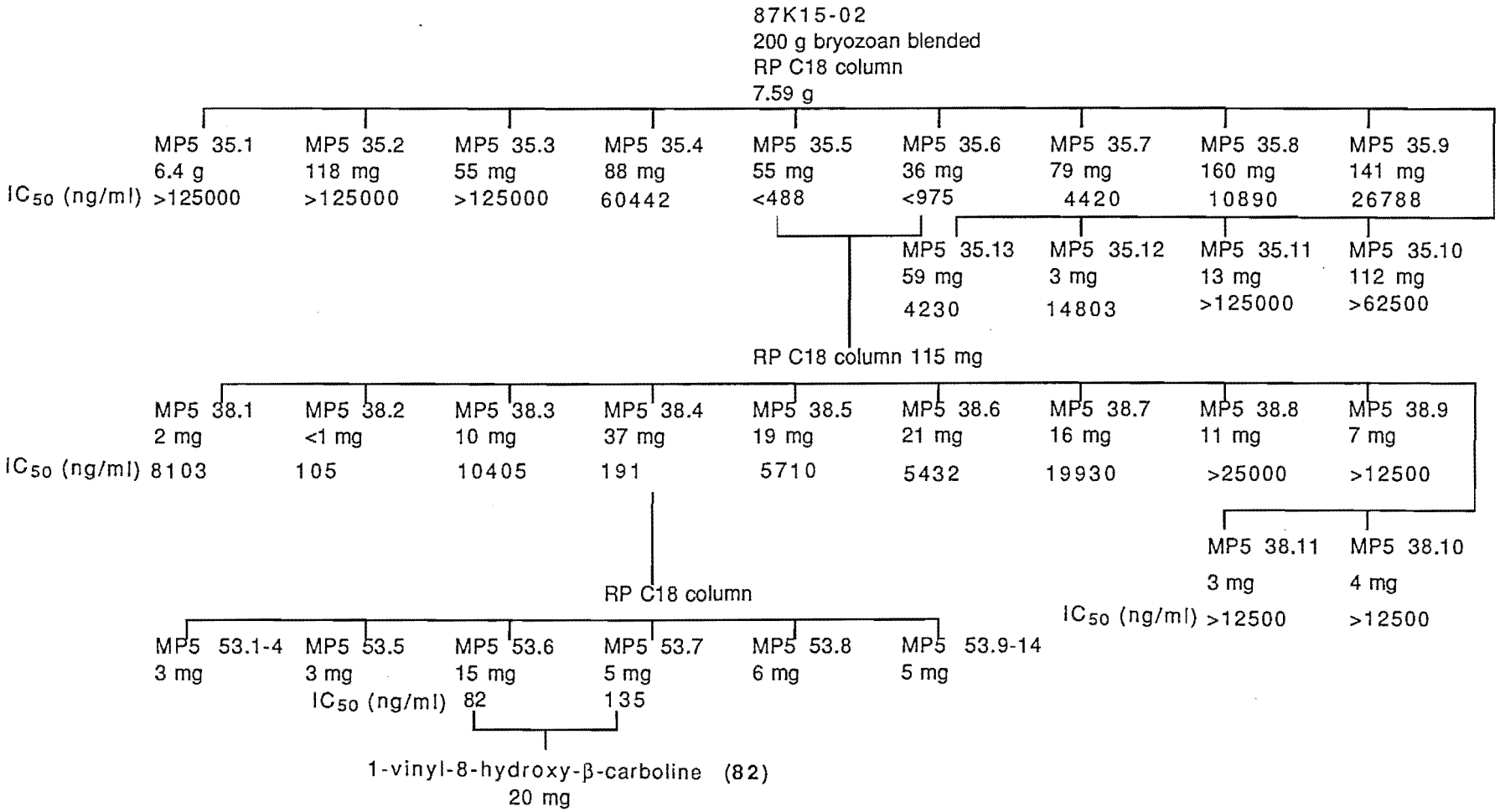


Figure A.1: Separation tree for *Cribricellina cribraria*

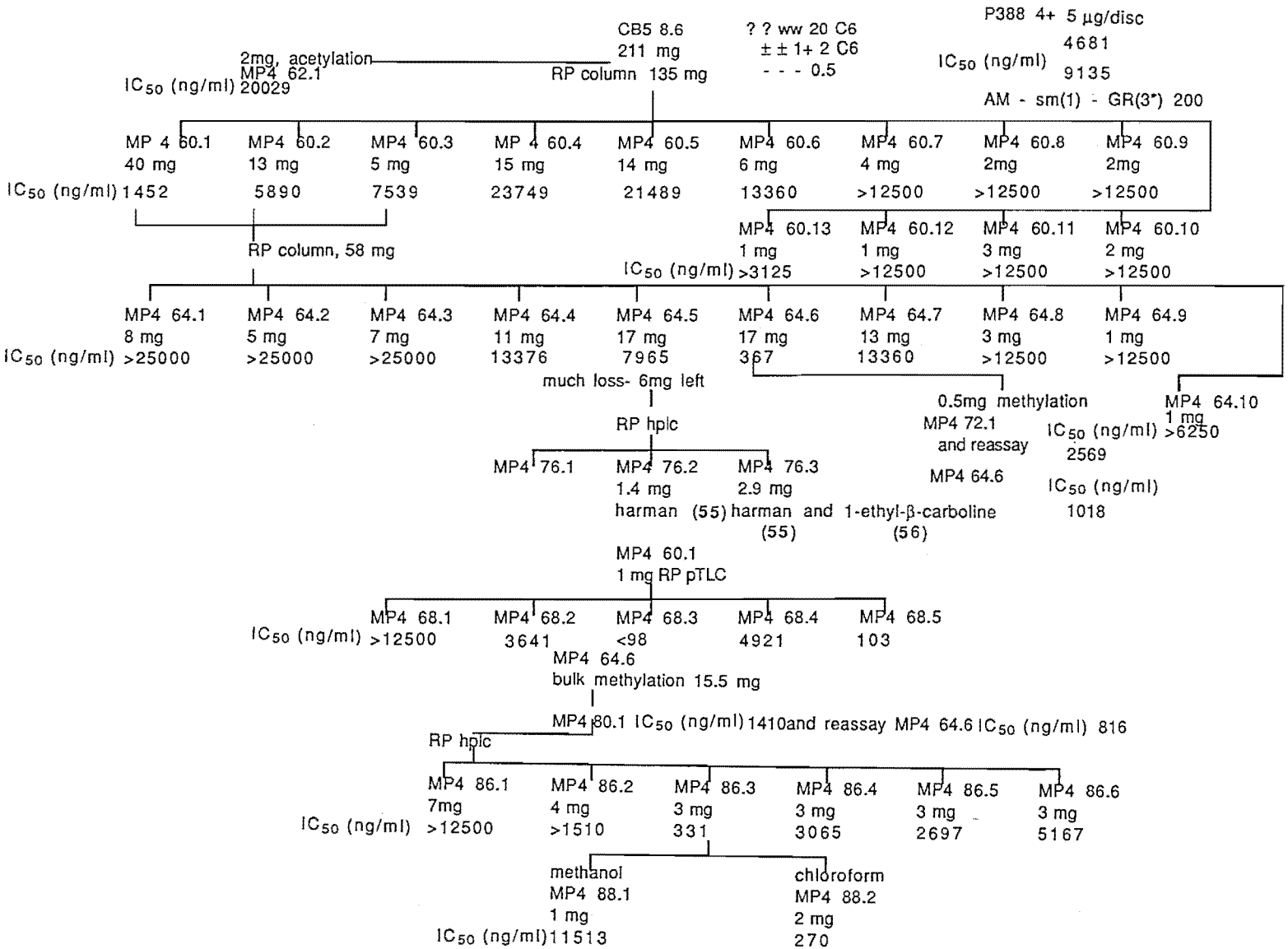


Figure A.2: Separation tree for *Margaretta barbata*

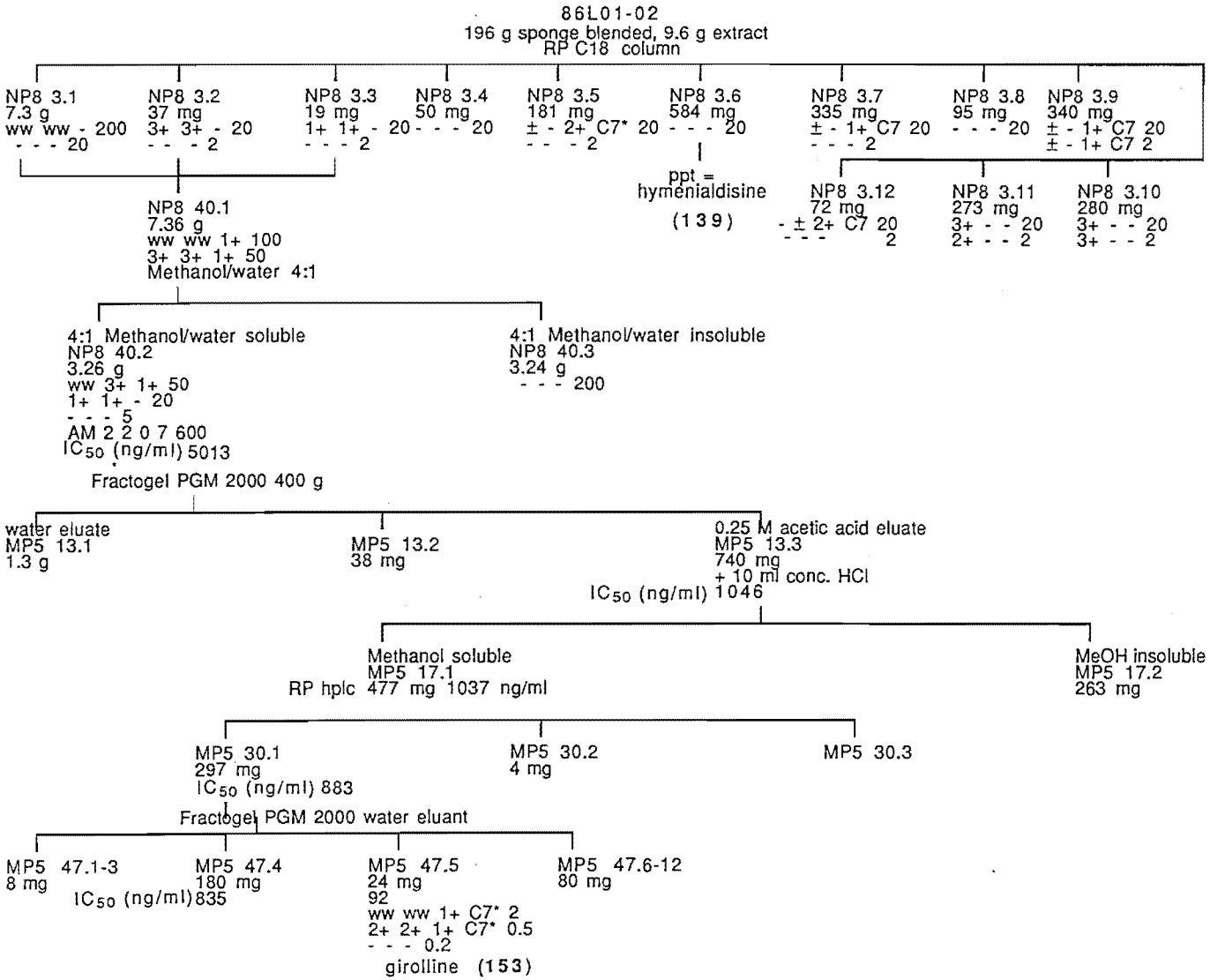


Figure A.3: Separation tree for *Axinella* sp. 2

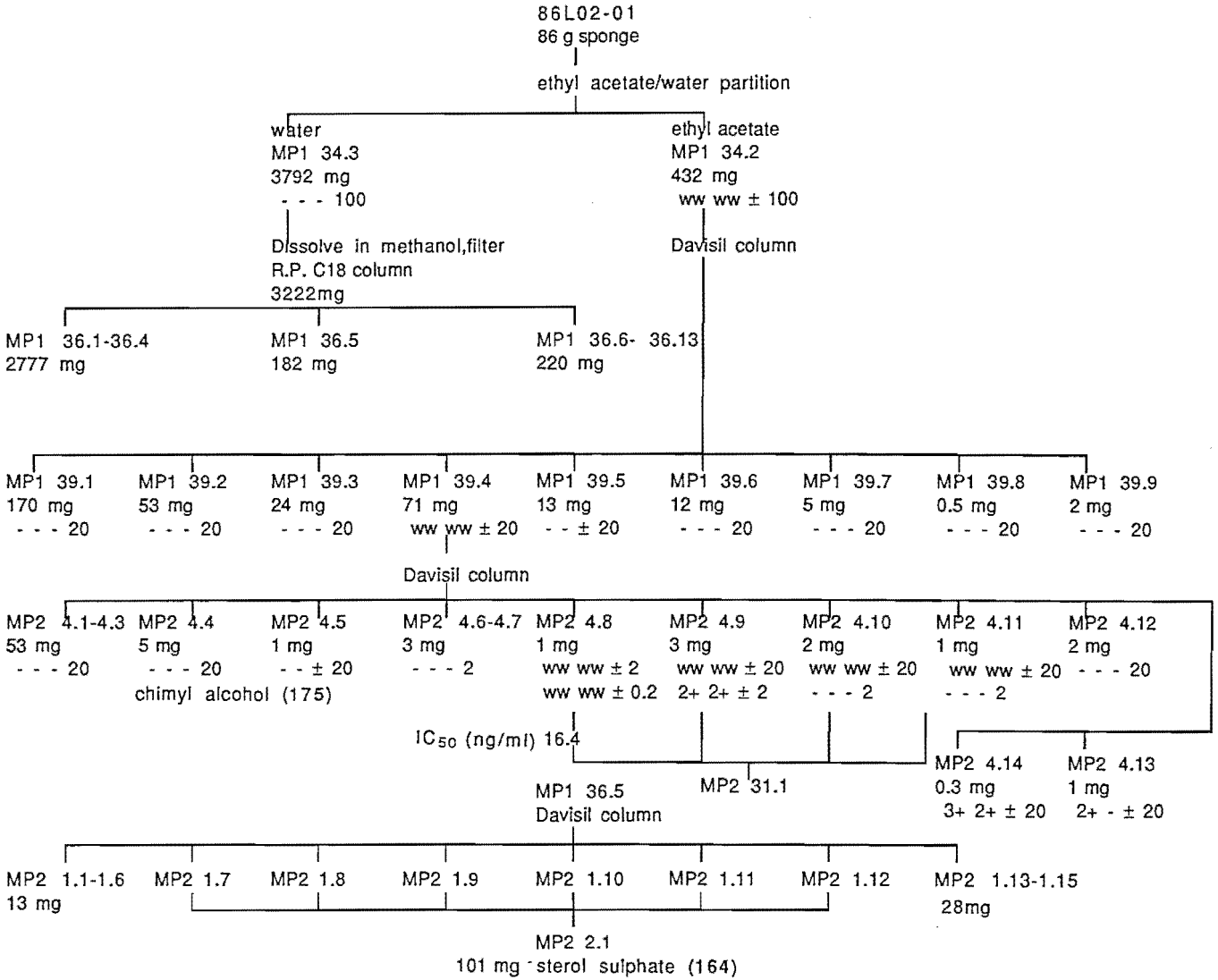


Figure A.4: Separation tree for *Stylopus australis*

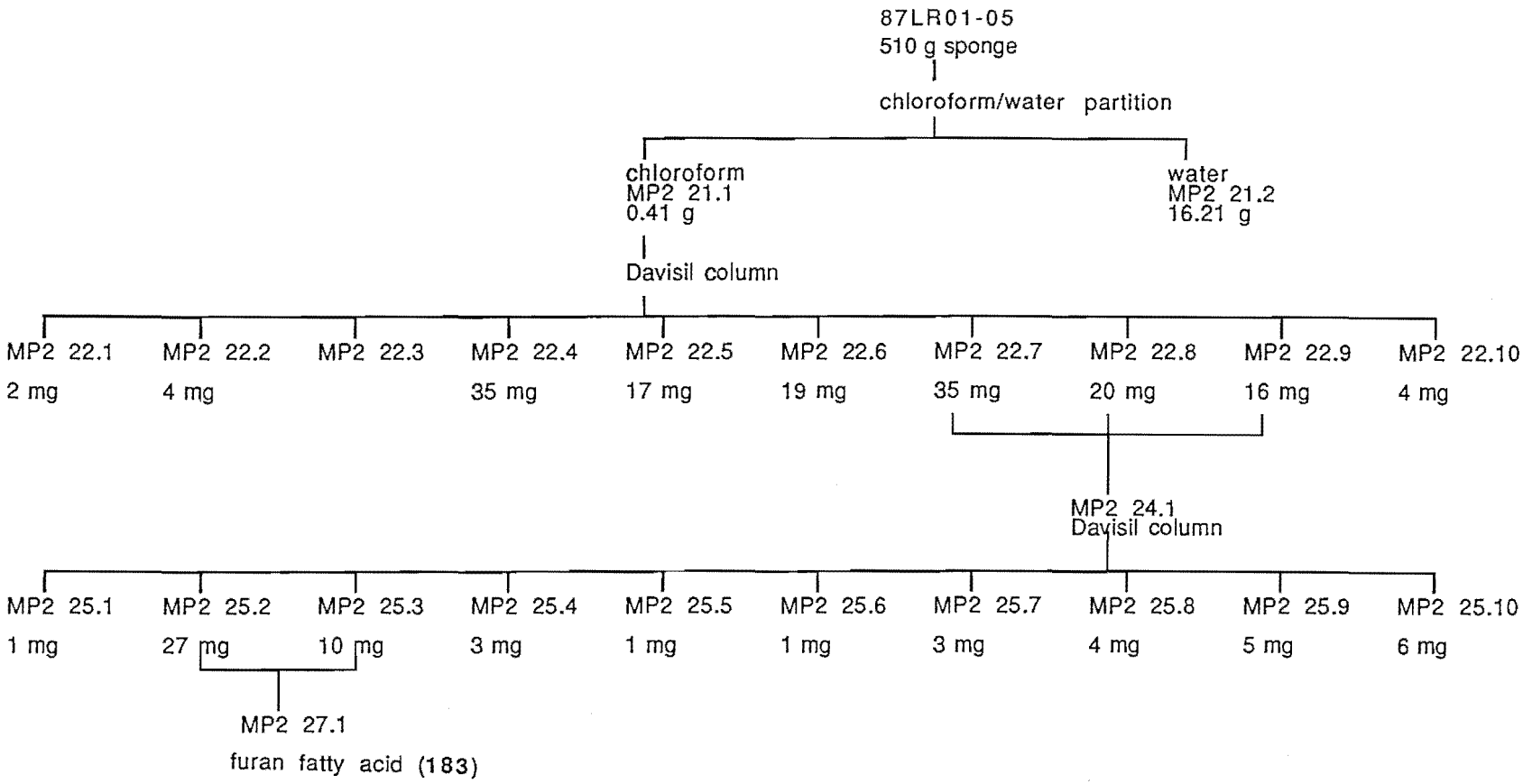


Figure A.5: Separation tree for *Hymeniacidon hauraki*

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